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# Mechanisms of inflammatory endothelial dysfunction

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Thesis submitted for the degree of PhD





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# Abstract

**Background**

Inflammation is important in the development, progression and acute complications of atherosclerosis. Increased levels of inflammatory markers in humans predict the occurrence of future atherothrombotic events. Recognised risk factors for atherosclerosis have been shown to cause endothelial dysfunction, characterised by impaired endothelial nitric oxide-dependent vasodilator responses. Whether a similar mechanism underlies the effects of inflammation is not fully understood. This work explores this relationship, with a particular emphasis upon changes in the endothelial nitric oxide synthesis pathway.

**Methods**

*In vitro* experiments were performed using organ bath pharmacology and western blotting to determine the effects of incubation of vascular tissue with high concentrations of the inflammatory marker C-reactive protein. An *in vivo* model of inflammation, using typhoid vaccination in healthy volunteers, was used to explore the time course of the vascular responses in comparison to the inflammatory markers. Further *in vivo* experiments involved selectively supplementing components of the nitric oxide pathway to determine if any were the cause of the endothelial dysfunction.

**Results**

The work showed that acute inflammation caused transient endothelial dysfunction and that this was associated with increased oxidant stress. Supplementation experiments showed no substrate deficiency, rather that there was increased consumption of both nitric oxide and the co-factor tetrahydrobiopterin required for its production. *In vitro* and *in vivo* experiments indicated that, although C-reactive protein was a strong predictor of future events, it did not itself cause endothelial dysfunction, and in fact may be associated with an improvement in nitric oxide bioavailability through increased tetrahydrobiopterin.

**Conclusions**

Overall, inflammation is associated with transient endothelial dysfunction and this may be important in the destabilisation of atherosclerotic disease and lead to clinical events. This change seems to be mediated by an increase in oxidant stress, which then has a number of related detrimental effects upon the nitric oxide pathway. However the acute phase marker, C-reactive protein, is only a marker of the underlying process and does not in itself appear to be causal in the development of endothelial abnormalities. Further work will be needed to more clearly characterise these effects and determine their clinical relevance.

# Contents

# Figures

# Tables

# Publications

Papers arising from this work

Inflammation-induced endothelial dysfunction involves reduced nitric oxide bioavailability and increased oxidant stress

**B Clapp**, A Hingorani, R Kharbanda, V Mohamed-Ali, J Stephens, P Vallance, R MacAllister

Cardiovascular Research 2004. 64(1):p172-178

Inflammation and endothelial function: Direct vascular effects of human C-reactive protein on NO bioavailability

**B Clapp**, G Hirschfield, C Storry, R Gallimore, R Stidwell, M Singer, J Deanfield, R MacAllister, P Vallance, M Pepys, A Hingorani

Circulation (in press)

Published abstracts arising from this work

Inflammation impairs NO-mediated arterial dilatation in humans

**B Clapp**, R MacAllister, R Kharbanda, A Hingorani, P Vallance

Circ 2001; 104(17) suppII p152

Inflammation impairs NO-mediated arterial dilatation in humans

**B Clapp**, R MacAllister, R Kharbanda, A Hingorani, P Vallance

J Human Hyp 2001; 15(12): p887-901

Human CRP has direct effects on the vascular reactivity of isolated rat aorta

**B Clapp**, G Hirschfield, R McAllister, J Gallimore, P Vallance, M Pepys, A Hingorani

J Human Hyp 2002; 16(12): p879-900

CRP alters vascular reactivity by increasing NO production

**B Clapp**, G Hirschfield, R McAllister, J Gallimore, P Vallance, M Pepys, A Hingorani

JACC 2003; 41(6) supp A: p319A

Reactive oxygen species contribute to inflammation induced endothelial dysfunction

**B Clapp**, A Hingorani, R MacAllister, P Vallance

JACC 2003; 41(6) supp A: p240A

Reactive oxygen species contribute to inflammation induced endothelial dysfunction

**B Clapp**, A Hingorani, R MacAllister, P Vallance

Heart 2003; 89 suppl: pA29

C-reactive protein values do not correlate with endothelial dysfunction during experimental inflammation in humans

**B Clapp**, G Hirschfield, C Storry, R MacAllister, J Deanfield, M Pepys, A Hingorani: J Human Hyp 2003

# Abbreviations

| | |
|---|---|
| ABTS | 2,2-azino-bis-3-ethylbensthiazoline-6-sulfonic acid |
| ACEi | Angiotensin converting enzyme inhibitor |
| ACh | Acetylcholine |
| ADMA | Asymmetric dimethylarginine |
| ANOVA | Analysis of variance |
| AMP | Adenosine monophosphate |
| $AT_1$ | Angiotensin receptor 1 |
| AUC | Area under the curve |
| $BH_4$ | Tetrahydrobiopterin |
| $BH_2$ | Dihydrobiopterin |
| BK | Bradykinin |
| BMI | Body-mass index |
| CABG | Coronary artery bypass grafting |
| cGMP | Cyclic guanodine monophosphate |
| CMV | Cytomegalovirus |
| COX | Cycloxygenase |
| CRP | C-reactive protein |
| DMEM | Dulbecco's modified Eagles medium |
| EBV | Ebstein Barr Virus |
| EDHF | Endothelium-derived hyperpolarizing factor |
| ER | Endoplasmic reticulum |
| ESR | Erythrocyte sedimentation rate |
| ET | Endotheln |
| FAD | Flavine adenine dinucleotide |
| FMD | Flow-mediated dilatation |
| FMN | Flavin mononucleotide |
| GFRP | GTPCH-1 feedback regulatory protein |
| GTN | Glyceryl trinitrate |
| GTPCH-1 | GTP cyclohydrolase -1 |
| HCAEC | Human coronary artery endothelial cells |
| HDL | High density lipoprotein |
| HPLC | High pressure liquid chromatography |
| ICAM | Intercellular adhesion molecule |
| IgG | Immunoglobulin G |
| IL-* | Interleukin-* |
| IL-1Ra | Interleukin-1 receptor anatagonist |
| IMA | Internal mammary arteries |
| Km | Michaelis constast |
| LDL | Low density lipoprotein |
| L-NAME | Nitro-L-arginine methyl ester |
| L-NMMA | $N^G$-monomethyl-L-arginine |
| LPa | Lipoprotein a |
| LPS | Lipopolysaccharide |
| MAS | Methoxyacetylserotonin |
| MCP-1 | Monocyte chemoattractant protein-1 |
| MCSF | Monocyte-colony stimulating factor |

| | |
|---|---|
| MMP | Matrix metalloproteinase |
| mRNA | Messenger ribonucleic acid |
| MTT | Thiazolyl blue tetrazolium blue |
| NADH | Nicotinamide adenine dinucleotide |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| NE | Norepinephrine |
| NED | Naphthylethylenediamine |
| NO | Nitric oxide |
| NOS | Nitric oxide synthetase |
| PBS | Phosphate buffered saline |
| PBST | Phosphate buffered saline Tween |
| PE | Phenylepherine |
| $PGI_2$ | Prostaglandin $I_2$ |
| PIA-1 | Plasminogen activator inhibitor 1 |
| PECAM | Platelet-endothelial cell adhesion molecule |
| PNP | 4-nitrophenyl phosphate |
| PPAR | Peroxisome proliferator-activated receptor |
| SD | Standard deviation |
| SEM | Standard error of the mean |
| sGC | Soluble guanylate cyclase |
| SLE | Systemic lupus erythematosis |
| SNP | Sodium nitroprusside |
| TAOS | Total antioxidant status |
| TEMED | NNNN-tetramethylethylenediamine |
| TNFα | Tumour necrosis factor α |
| tPA | Tissue plasminogen activator |
| TX | Thromboxane |
| UAC | Urinary albumin/creatinine ratio |
| VCAM | Vascular cell adhesion molecule |
| VTI | Velocity time intergral |
| vWF | Von Willebrand |

# 1 Introduction

## 1.1 Epidemiology of Atherosclerosis

Atherosclerosis and its complications account for 15 million deaths per annum worldwide, and within 50 years it is predicted that atherosclerosis will overtake infectious diseases to become the single commonest cause of death([1;2]). Each year 110,000 people in the United Kingdom die from coronary disease and nearly half of these are less than 75 years old. 90,000 patients have a myocardial infarction annually and stroke remains the largest cause of severe disability, and the third most common cause of death, in the United Kingdom. Atherosclerosis has a long pre-clinical phase, with early lesions being evident as young as six months old ([3]). Although it is a disease that originates in early life, its clinical burden is most heavy upon the late middle and old aged. As a consequence it has a huge social and economic burden, and understanding the mechanisms of atherosclerosis remains a vital prelude to the discovery of new treatments.

## 1.2 Pathology of Atherosclerosis and the Natural History of Plaque Formation

The first stages of the development of atherosclerosis are poorly understood in humans. Animals treated with a high lipid diet initially deposit small lipoprotein particles in the intima, which coalesce to form fatty streaks([4]). Initially intimal thickening occurs in regions of the vasculature prone to the development of atherosclerosis, such as bifurcation points([5]). This may be followed by lipid accumulation and development of fatty streaks. With increasing age more

complex lesions develop, characterised by the presence of extracellular lipid, fibrosis and accumulation of lymphocytes, smooth muscle cells and macrophages.

Lesions are divided into precursor (type I-III) and advanced lesions (IV-V) according to their structure([6]). Type I lesions have isolated scattered macrophages containing lipid droplets, whereas type II lesions contain macrophage foam cells in layers with lipid deposits within the intimal smooth muscle cells and T-lymphocytes; these are seen macroscopically as fatty streaks. Type III lesions differ importantly in that the lipid pool is now extracellular. Once this coalesces to a tight "lipid core" the lesions are classed as type IV and the final lesion type is present once fibrous tissue becomes a significant feature (although these may again be subdivided according to the presence of calcification [Vb] or relative dominance of fibrosis [Vc]).

Although increasing lesion complexity is associated with greater bulk, the development of vessel wall remodelling allows the incorporation of large lesions without necessarily impeding flow([7]). Post-mortem and intra-vascular ultrasound studies suggest that the lesions which lead to atherothrombotic events differ structurally from those leading to luminal stenosis; they are predominantly located within the intima and media with outward expansion (positive remodelling), and have an higher lipid content containing increased numbers of inflammatory cells([8-10]). Lesions that demonstrate high degrees of stenosis may occur due to rupture, thrombosis and subsequent rapid growth of intramural plaques with resultant smooth muscle proliferation and show a large number of areas of healed

plaque disruption([11]). Such a process may explain the phasic rather than linear progression of lesions seen in repeat angiography studies([12]).

Atherosclerosis is a prolonged process that starts early in life. The human post-mortem studies of Stary and McGill have demonstrated that by the third decade of life 20% of individuals will have advanced lesions present in the coronary circulation([3;13]). In childhood, type I and II lesions are present, with type III lesions appearing around puberty and type IV lesions in the third decade. Recently, data from intra-vascular coronary ultrasound studies have shown that nearly 17% of donor hearts from individuals aged less than 20 years of age have detectable atherosclerosis, and this proportion is nearly 50% in donors over 50 years old([14]). Further, in individuals where lesions are demonstrable angiographically widespread atheroma can be detected by ultrasound in other apparently normal parts of vessels([15]).

## 1.3 Theories of Pathogenesis of Atherosclerosis

The three fundamental processes in the pathogenesis of atherosclerosis are the accumulation of lipid in the vessel wall, smooth muscle proliferation and inflammation.

Virchow's *imbibition hypothesis* proposed that the cellular proliferation seen in the intima was a form of 'low-grade inflammation' as a reaction to infiltration, or imbibition, of proteins and lipids from the blood. The *encrustation theory* proposed by von Rokitansky postulated that small thrombi composed of platelets,

fibrin, and leukocytes collect over sites of endothelial injury, which then organise with smooth muscle proliferation to form atherosclerotic plaques([16;17]).

Benditt and Benditt and others have demonstrated in human plaques, by use of X-chromosome inactivation according to the Lyon hypothesis, that the intimal cell proliferation is monoclonal([18]). This is not due to allele selection and has not been demonstrated in other animals, at least in part due to methodological difficulties. This monoclonality could indicate three possibilities; firstly the insult could be very localised leading to one clone expanding, secondly the expanding "plaque smooth muscle" cells could preferentially over-proliferate due to infection with a virus or other pathogen or thirdly the proliferating cells could be genetically distinct and favour growth([19]). The details and importance of this mechanism in atherosclerosis are not clear particularly as monoclonal expansion has been detected in regions of normal intima and media([20]).

Preferential retention of lipoproteins has been suggested as the key step in atherosclerosis. This is based on the observation that there is adequate influx of lipid into all cells, whereas the failure to excrete it is characteristic of plaques, thus leading to excess retention in areas of atheroma. This occurs at the points of shear stress that typically demonstrate plaque development and it has been proposed that retained lipid is then oxidised and acts as the stimulus for other aspects of atherosclerosis([21]).

In the 1970s Ross suggested a mechanism whereby lipid deposits might gain access to the vascular wall, the so-called "the response-to-injury hypothesis"([22]).

This proposes that the initiation of atherosclerosis involves endothelial denudation, allowing adhesion of platelets, monocytes and leukocytes to the vessel and increasing permeability to plasma constituents. As a further modification to this theory, the endothelium need not be denuded, rather merely dysfunctional([23]) to act as a substrate for atherosclerosis. However, irrespective of the details of the late mechanisms of atherosclerosis, a common theme in each of these theories is the presence, early in the pathogenesis of atherosclerosis, of endothelial dysfunction.

## 1.4 Physiology of endothelium

The endothelium is the single layer of cells that lines the luminal surface of blood vessels and regulates numerous blood vessel functions including vascular tone, cell adhesiveness, and coagulation by the generation of local mediators. These include vasodilators, of which the most important are nitric oxide (NO), prostacyclin ($PGI_2$), and endothelium derived hyperpolarising factor (EDHF). The endothelium, also generates a number of locally acting vasoconstrictors including thromboxane (TX) and endothelin (ET).

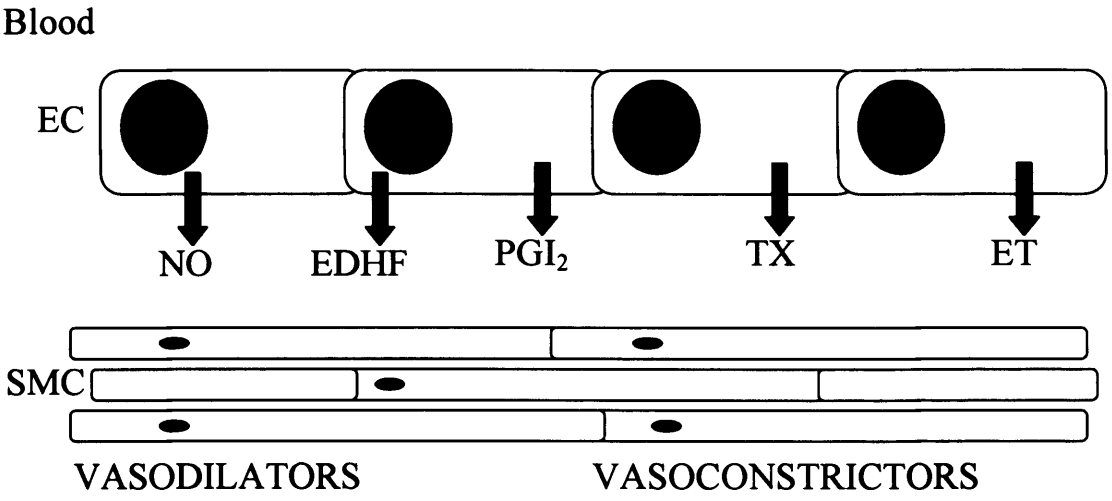



**Figure 1-1: Endothelial vasodilators and vasoconstrictors**

(EC = endothelial cell; SMC = smooth muscle cell; NO = nitric oxide; EDHF = endothelial derived relaxing factor; PG = prostaglandin; TX = thromboxane; ET = endothelin)

### 1.4.1 Vasoactive mediators

In health, the endothelium favours the production of vasodilators with the relative importance of each pathway being dependent on the vasculature studied. This section will describe the important vasoactive pathways.

#### 1.4.1.1 Nitric Oxide

Nitric oxide (NO) is a freely diffusible gas generated from the amino acid L-arginine via a five-electron oxidation of a guanidino-nitrogen by nitric oxide synthase. Nitric oxide synthase (NOS) has three isoforms, named according to their original description; neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). eNOS is probably constitutively expressed within caveolae in the endothelial plasma membrane bound to calveolin([24]) and releases NO following stimuli that act upon the endothelial cell surface. NOS is a

myristoylated multi-domain enzyme consisting of an N-terminal oxygenase domain containing binding sites for heme, L-arginine and tetrahydrobiopterin ($BH_4$) and a reductase domain containing binding sites for flavine adenine dinucleotide (FAD), reduced nicotinamide adenine dinucleotide phosphate (NADPH), flavin mononucleotide (FMN) and calmodulin([25]).

At rest, although some eNOS produces a tonic level of NO, the majority of eNOS is inactive, but an increase in intracellular calcium activates calmodulin that displaces the calveolin, and possibly also a polypeptide inserted into the FMN domains([26]), and activates the enzyme([27]). Intracellular calcium is increased by receptor-mediated agonists, such as acetylcholine (ACh) and bradykinin (BK) and physical stimuli, such as shear stress. The calmodulin binding allows NOS activation by enabling the reductase domain of the enzyme to transfer electrons to the oxygenase domain where the heme iron can bind $O_2$ and catalyse the stepwise production of NO([28]). By contrast iNOS is neither calcium nor calmodulin dependent, or constitutively expressed; instead its expression can be induced by inflammatory stimuli.

The active form of NOS that preferentially generates NO is a dimer of two identical subunits. The maintenance of this dimer is a process that requires the presence of adequate concentrations of $BH_4$, the exact actions of which are unclear, though it probably acts in an allosteric and redox fashion, at least in part by facilitating L-arginine binding([29;30]). In the absence of adequate $BH_4$, or L-arginine, eNOS changes its phenotype to preferentially produce superoxide anion ($O_2^-$)([31]) which will bind to NO producing peroxynitrite that is potentially harmful

to the vasculature. Superoxide can also further deplete $BH_4$ by oxidation to dihydrobiopterin and thereby worsen the uncoupling of eNOS - thus producing a cascade of deleterious effects.

Once generated NO acts both within the lumen of the vessel, where it is a potent inhibitor of leukocyte adhesion and platelet activation([32]), and also on surrounding smooth muscle cells and tissues. Vascular NO is involved in the control of angiogenesis both as a direct messenger and also by its control of growth factor expression([33]). Within adjacent smooth muscle cells it increases soluble guanylate cyclase (sGC) activity by up to four hundred-fold([34]), following binding of NO to two heme molecules and causing a conformational change([35]). Once activated sGC leads to the production of cyclic 3', 5' guanosine monophosphate (cGMP) which reduces intracellular calcium and thereby causes smooth muscle relaxation([36]).

In addition to stimulated increases in NO production basal eNOS activity is involved in the maintenance of vascular tone. Blockade of NOS activity systemically with $N^G$-monomethyl-L-arginine (L-NMMA) in healthy human volunteers resulted in an increase in blood pressure([37]), and blockade of NOS activity in the forearm reduced forearm blood flow([38]), indicating the importance of basal NO release. Endogenous basal NO-production has been demonstrated in coronary, pulmonary and systemic vasculature, indicating its importance in the regulation of flow in numerous vascular beds([39;40]).

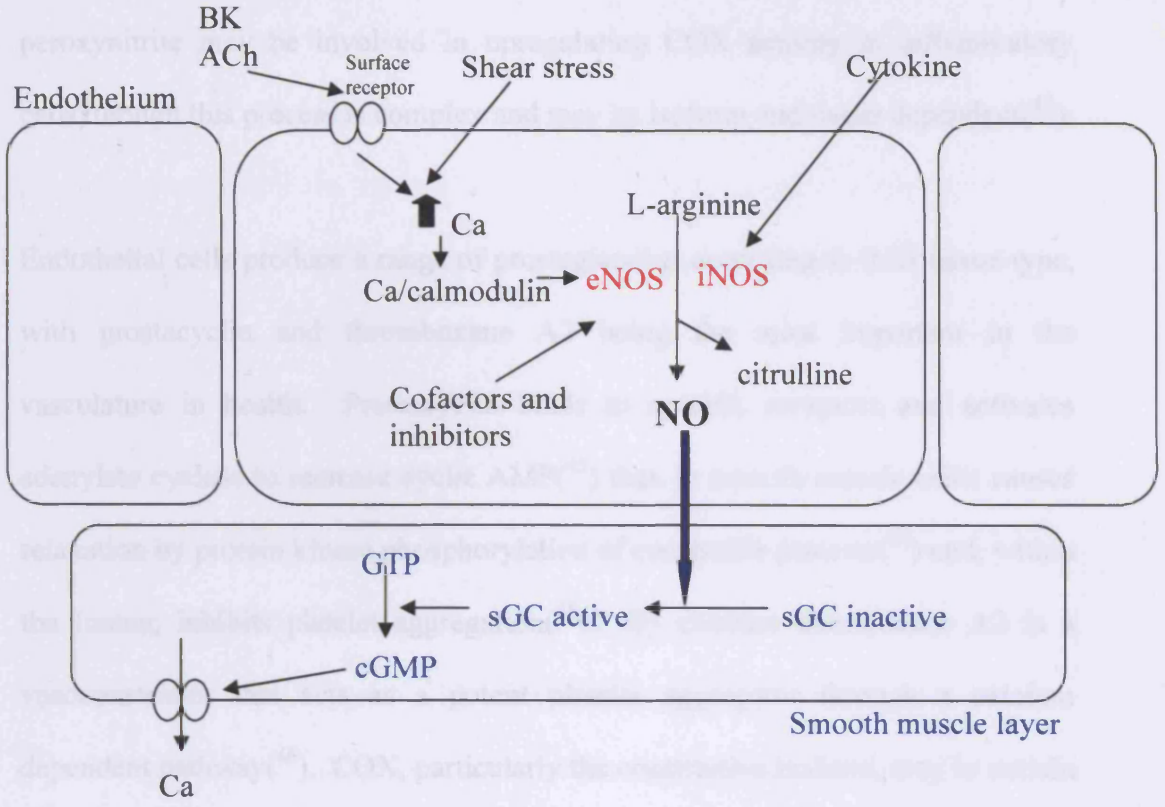



**Figure 1-2: Intra and intercellular NO signalling**

This diagram shows the activation and intracellular effect of nitric oxide. Bradykinin (BK) and acetylcholine (ACh) or shear stress increase intracellular calcium in endothelial cells, activate calmodulin and increase eNOS activity. Alternatively cytokines act, in a calcium-independent way, to increase nitric oxide through iNOS activation. Nitric oxide diffuses to smooth muscle cells (shown in blue) and by activating gaunylate cyclase causes a reduction in calcium concentrations and muscle relaxation.

### 1.4.1.2 Prostaglandins

Prostaglandins are produced from arachidonic acid, which is released from membrane lipids by phospholipase $A_2$ and metabolised by cyclo-oxygenase (COX)([41]). COX has two isoforms; COX-1, which is constitutively expressed,

and COX-2 that is induced by cytokines and differs by a single amino acid substitution in the enzymatic site. Nitric oxide or its breakdown product peroxynitrite may be involved in upregulating COX activity in inflammatory cells, though this process is complex and may be isoform and tissue dependent([42]).

Endothelial cells produce a range of prostaglandins according to their tissue type, with prostacyclin and thromboxane A2 being the most important in the vasculature in health. Prostacyclin binds to specific receptors and activates adenylate cyclase to increase cyclic AMP([43]) that, in smooth muscle cells, causes relaxation by protein kinase phosphorylation of contractile proteins([44]) and, within the lumen, inhibits platelet aggregation([45]). By contrast thomboxane A2 is a vasoconstrictor that acts as a potent platelet aggregator through a calcium dependent pathway([46]). COX, particularly the constitutive isoform, may in certain circumstances also be an important source of oxygen free radicals([47]).

In healthy individuals COX-1 inhibition with aspirin (and other non-steroidal anti-inflammatory agents) has been shown to reduce blood flow in human forearm resistance vessels([48]). This is suggestive of a role for prostanoids in vascular tone in the forearm, a phenomenon that has also been demonstrated in the coronary circulation([49]). Anti-inflammatory doses of aspirin have been shown to prevent inflammation induced endothelial dysfunction in the forearm, though a direct local effect of prostanoid inhibition could not be demonstrated([50]).

### 1.4.1.3 Endothelium-derived hyperpolarizing factor (EDHF)

In addition to NO and prostaglandins, a contribution to vasodilatation has been attributed to the unidentified EDHF. EDHF causes hyperpolarisation by a

potassium channel-dependent mechanism that leads to vasorelaxation. Not only is the character of EDHF uncertain, it may in fact vary between different vessels and has been "identified" variously as potassium ions, cytochrome P450 metabolites, C-type natriuretic peptide and myo-endothelial gap junctions([51;52]). The relative importance of EDHF is thought to increase as the vessel diameter decreases, which correlates with an increase in the prevalence of gap junctions([53;54]).

Human studies of the effects of EDHF are made difficult due to the uncertainty about its identity and the toxicity of many of the potassium channel blockers used in its study in animals; there has been some work with barium, ouabain and miconazole that may inhibit EDHF-dependent effects. Dawes et al have shown a reduction in basal flow and dilatation to 50-200μmol/min potassium infusion in the human forearm by plethysmography following the infusion of barium chloride and ouabain at doses thought to selectively affect inward-rectifier potassium channels([55]). Inhibition of cytochrome-P450 metabolism, with miconazole, has also shown a reduction in NO-independent vasodilatation in the human forearm to bradykinin([56]). In humans with hypertension an increased NO-independent vasodilator effect of bradykinin has been demonstrated, which is inhibited by ouabain, suggesting that the relative importance of non-NO pathways may change in diseased vessels([57]).

#### 1.4.1.4 Endothelin

Endothelins are a family of 21-amino acid vasoconstrictor peptides encoded by three genes, of which the predominant vascular form is endothelin-1. This acts in a paracrine and autocrine manner on two receptors, $ET_A$ and $ET_B$, present on both endothelial cells and adjacent smooth muscle([58]). The primary effects of $ET_A$

receptor activation are to cause vasoconstriction, cell proliferation and hypertrophy([59]), though, in certain circumstances, stimulation of $ET_B$ can increase production of NO and prostacyclin – leading to vasodilatation([60]).

Endothelin infusion intra-brachially reduces human forearm blood flow in healthy volunteers([61]), whereas antagonists cause an increase in flow suggesting a role for basal and stimulated endothelin release in determining vascular tone([62]). Similar effects on basal tone have been demonstrated in human coronary arteries([63]). In individuals with essential hypertension, endothelin and its antagonists have been shown, by some investigators, to have an increased effect compared to controls in both the hand vein and resistance vasculature (by plethysmography), indicating a potential increase in importance in diseased vessels([64;65]). In addition to its tonic effects, endothelin has been shown to be chemoattractant for macrophages and monocytes and may be involved in smooth muscle mitogenesis([66]), indicating a potential role in the development of atherosclerosis. In support of this, raised levels of endothelin are present in the plasma and tissue in proportion to the plaque burden of advanced atherosclerosis and in individuals with early atherosclerosis and cardiac risk factors([67;68]).

### 1.4.2 Regulation of circulating cells and cell adhesion

In addition to the release of paracrine substances described, the endothelium acts upon circulating cells and contributes to the regulation of their tendency to adhesion by a number of additional mechanisms. Early atherogenesis may be dependent upon the ability of the endothelium to capture leukocytes from the circulating blood, which then transform into foam cells. This is dependent upon

three types of molecule expressed on the surface of endothelial cells – selectins, integrins and immunoglobulins.

### 1.4.2.1 Selectins

Selectins are transmembrane glycoproteins containing an amino-terminal calcium-binding lectin domain that mediate the first step in leukocyte adhesion at the site of inflammation and injury([69]). There are three types of selectin: E-selectin is synthesised in response to interleukin-1, tumour necrosis factor, oxygen radicals and bacterial toxins([70]) and found primarily on endothelial cells; P-selectin is stored within Weibel-Palade bodies in endothelial cells and α-granules of platelets and can be very rapidly expressed following exposure to histamine or thrombin([71]); L-selectin is basally expressed on leukocytes([72]). The major ligand for all the selectins is a specific sialyl-Lewis$^x$-type glycoprotein on circulating neutrophils and platelets.

### 1.4.2.2 Integrins

These are heterodimeric molecules consisting of non-covalently bound α and β subunits, which are divided into families according to the subtype of β subunit. Although there are large numbers of variations, three groups are most important in atherogenesis ($\beta_1$-$\beta_3$)([73]). The "very late appearing antigen" intergrins, $\beta_1$ family, are the group expressed on endothelial cells and primarily mediate cell adhesion to extracellular matrix proteins such as collagen and laminin. Although expressed only on leukocytes, $\beta_2$ intergrins are involved in the movement of cells across the endothelium. The endothelial ligand is dependent upon the α subunit, either being a member of the immunoglobulin family or other large matrix proteins([74]).

### 1.4.2.3 Immunoglobulin gene superfamily

The immunoglobulin gene superfamily consist of a series of 90-100 amino acid domains that act as endothelial ligands for integrins expressed by leukocytes and platelets. These are involved in cell-cell interaction and signal transduction. The most important members are vascular cell adhesion molecule (VCAM-1), intercellular adhesion molecules (ICAM-1 and 2) and platelet-endothelial cell adhesion molecule (PECAM). Both ICAM and VCAM expression (the latter of which is not expressed on endothelial cells basally) is upregulated by cytokines, primarily tumour necrosis factor and interleukin-1, leading to increased leukocyte adhesion([75]). Recognised cardiovascular risk factors, such as oxidised low-density lipoprotein have been shown to increase ICAM-1 expression([76]). In animal models VCAM-1 expression occurs early after the initiation of an atherogenic diet([77]) and VCAM knock-out mice have reduced atherosclerotic lesion development([78]).

## 1.4.3 Regulation of coagulation and fibrinolysis

The endothelium is critically involved in haemostasis and this is central to the progression of atherosclerosis and the development of acute thrombotic syndromes. Primary haemostasis is initiated by injury to the vascular wall with the formation of a "platelet plug" and represents a failure in the balance between coagulation and fibrinolytic pathways. Under normal conditions the endothelium is anticoagulant by its regulation of enzymes within the coagulation cascade, most notably thrombin.

Thrombin production is kept in check by antithrombin III bound to the endothelial surface on heparan sulphate proteoglycans([79]). In the presence of damaged endothelium, thrombin acts to convert fibrinogen to fibrin and promote clot formation. In health thrombin also binds to a specific receptor, thrombomodulin, and upregulates the protein C and S pathways that degrade key clotting factors and reduce the activity of plasminogen activator inhibitor 1 (PAI-1), the predominant inhibitor of the fibrinolytic system([80]). Inflammatory mediators and bacterial toxins downregulate the expression of thrombomodulin, and upregulate PAI-1 expression and vessel injury leads to further reductions by cleavage of thrombomodulin from the endothelium.

As a result of reduced PAI-1 production the endothelium favours fibrinolysis driven by tissue plasminogen activator (tPA) that increases conversion of plasminogen to plasmin. tPA activity is dependent upon PAI-1 levels as they form a stable inactive complex. NO enhances the activity of the fibrinolytic pathway by increasing tPA expression, as well as acting directly to reduce platelet aggregation by a prostaglandin-dependent pathway.

The endothelium also generates a number of pro-coagulant mediators, von Willebrand factor (vWF), fibronectin, tissue factor and thrombospondin, which are important in the haemostatic response. Thrombospondin promotes platelet aggregate stabilisation and depresses fibrinolysis, fibronectin contributes to fibrin clot stabilisation and vWF acts, in part, to allow the initial adhesion and further aggregation of platelets([81]). Tissue factor is an integral membrane glycoprotein associated with phospholipids, not constitutively expressed on endothelial cells,

which binds factor-VII and triggers coagulation([82]). Following injury, or exposure to proinflammatory cytokines, the endothelium can rapidly alter its phenotype to favour the procoagulant and adhesive pathways described.

### 1.4.4 Endothelial monolayer integrity

The endothelium controls the extravasation of solutes, macromolecules and leukocytes. The healthy endothelium is usually a closed barrier to macromolecules that are taken up only by specific receptors or vesicular shunting. Following inflammation and angiogenesis the endothelium can become locally hyperpermeable. The degree of leak and its duration are dependent upon the stimulus, from transient pericellular gaps that develop following agonist stimulation to more prolonged defects following injury([83]). Increased permeability lasting 60-90 minutes is induced by thrombin in a mechanism involving both calcium/calmodulin and Rho kinase activation. As indicated in the section 1.4.3 active thrombin is increased by inflammation and may be important in the associated breakdown in membrane integrity([84]). Under basal conditions the effects of cyclic AMP on endothelial junctions and cyclic GMP on the inactivating thrombin maintain the integrity of the barrier([85]).

## 1.5 Endothelial Dysfunction and Atherosclerosis

In healthy endothelium the production of vasodilators and anticoagulants predominate. Following endothelial injury there is a change in the phenotype of the vessel wall leading to the promotion of the development of atherosclerosis and, potentially in mature lesions, the destabilisation of existing plaques. The

pivotal importance of endothelial dysfunction is demonstrated in both animal and human models of atherosclerosis.

### 1.5.1 Animal data

Animal models of atherosclerosis, and high atherosclerotic risk, demonstrate impaired endothelium-dependent relaxation of conduit([86-88]) and resistance([89;90]) vessels prior to significant anatomical changes. Generally endothelium-independent responses are not impaired, except in advanced lesions.

Later, anatomical changes involve an alteration in the orientation of endothelial cells, which appear more rounded, and changes in the subcellular cytoplasmic filaments([91]). There are macroscopic differences in the intima and media with relative thickening of the latter and ultrastructural changes in the internal elastic lamina to produce a more porous structure([92-94]).

### 1.5.2 Human data

Evidence exists for endothelial dysfunction in the presence of atherosclerosis and its risk factors in all arterial vessels that have been studied, suggesting it is an early event and that it may presage acute episodes. Endothelial dysfunction of the coronary and peripheral circulation correlates with the occurrence of cardiovascular events in patients at high risk of cardiovascular disease([95-99]). Individuals with multiple risk factors for cardiovascular disease, with a high Framingham Risk Score, also have an associated reduction in endothelial function([100]). Abnormalities in the coronary artery acetylcholine response, indicating endothelial dysfunction, have been shown in individuals without

structural changes on intra-coronary ultrasound scans, indicating alterations in function even before structure([101]). Given the invasive nature of assessing coronary endothelial function interest has focused on peripheral vessels as surrogates. Flow-mediated dilatation of human conduit arteries (a recognised test of endothelial function described later in this thesis; section 2.2.2) correlates with the extent of coronary atherosclerosis and the presence of coronary vascular abnormalities([102;103]).

In young humans, endothelial dysfunction has been shown in peripheral conduit vessels, by assessment of flow mediated dilatation, in individuals with high cholesterol, diabetes, a family history of heart disease and smokers([104-107]). Similar abnormalities have also been shown in resistance vessels, by forearm venous occlusion plethysmography, in patients with hypercholesterolaemia, insulin dependent diabetes and hypertension([108-111]) prior to the development of clinical atherosclerosis. Treatment of these risk factors for coronary artery disease with HMG CoA reductase inhibitors or LDL apheresis, or angiotensin-converting enzyme inhibition has been shown to improve endothelial dysfunction, an effect that may contribute to the reduction in cardiovascular events seen in randomised controlled trials([112-114]). In hypercholesterolaemia, the role the nitric oxide pathway plays in endothelial dysfunction is shown by its improvement with oral or intra-venous administration of the substrate for NO synthesis, L-arginine([106;115]), and in hypertension by the change in sensitivity to NO blockade with $N^G$-monomethyl-L-arginine([116]).

The loss of normal coronary responses to agonists, acetylcholine, serotonin and substance P, and to a flow stimulus have all been demonstrated in atherosclerotic vessels with the retention of a normal response to endothelium-independent vasodilators([39;103;117-120]). There is evidence of a progressive loss of responses, with a reduced response to acetylcholine and serotonin initially, followed by reduced response to substance P and then to flow. This suggests that different mechanisms may be affected at different stages of atherosclerosis([121-123]) .

In addition to changes in vasoactive function, abnormalities occur in the production of soluble factors by the endothelium in response to risk factors for atherosclerosis leading to a pro-coagulant phenotype([124]). Reduction in vasodilator function by FMD has been associated with an increase in von Willebrand factor([125;126]) which itself has been shown to be raised with hypercholesterolaemia and atherosclerosis([127;128]). Dysfunctional endothelium releases increased quantities of adhesion molecules, notably selectins and ICAM and VCAM, and these have also been correlated with the presence and activity of atherosclerotic disease([129;130]).

## 1.6 Inflammation in Atherosclerosis

There is increasing evidence that atherosclerosis is an inflammatory disease. The response-to-injury hypothesis proposes that inflammation initiates and promotes atherosclerosis through phenotypic changes in the endothelium leading to cell adhesion, inflammatory cell migration and thrombosis([23]). Evidence to support this theory comes from both animal models and multiple human diseases, as will be discussed below.

### 1.6.1 Animal data

Early studies have shown that inflammatory cells are major components of atherosclerotic plaques([131]). As previously discussed, inflammatory cytokines and oxidised LDL lead to up-regulation of VCAM-1, which then aids endothelial binding of inflammatory leukocytes. Other chemokines stimulate monocyte chemoattractant protein-1 (MCP-1) expression that then allows bound cells to infiltrate the vessel wall. The presence of inflammatory cells within the endothelium perpetuates the local inflammatory response and, under the influence of further cytokines (especially monocyte-colony stimulating factor; MCSF), these cells engulf lipids and form foam cells. Mice deficient in either MCP-1 or MCSF show reduced inflammatory cell uptake and atheroma even in the presence of an atherogenic diet([132;133]). Inflammatory cells may be important in the destabilisation of atherosclerotic plaques, with data from mouse models suggesting that inhibition of CD40 ligand, a potent signalling molecule between leukocytes, changes the character of the lesions to a more stable form([134]).

Experimental models also demonstrate that infection accelerates the development and severity of atherosclerosis. Chickens infected with Mareks disease virus, develop atherosclerosis that closely resembles human disease and is preventable by vaccination([135]). New Zealand white rabbits on a normal diet infected with *Chlamydia pneumoniae* develop intimal thickening and atherosclerotic lesions within the aorta that can be prevented by antibiotic treatment with azithromycin([136;137]). In cell culture studies *C. pneumoniae* has been shown to induce a murine macrophage cell line to change to a foam cell phenotype([138]). It is proposed that this is due to the ability of the chlamydial lipopolysaccharide to

stimulate lipid uptake by the macrophages, though, in contrast to other endotoxins, it does not induce a strong enough response to facilitate pathogen clearance.

Animal models have suggested that systemic inflammation can have generalised effects, additional to local changes, and that these can accentuate a prior predisposition to atheroma. In Apo-E knockout mice, infection with murine herpes virus 8([139]) or cytomegalovirus([140]) leads to accelerated atherosclerosis, as does endotoxin treatment of hypercholesterolaemic rabbits([141]). The use of *C. pneumoniae* in ApoE-deficient mice, however, has produced contradictory results dependent on the age of the animals, their sex and the exact characteristics of the infections used([142;143]).

Studies with double knock out animals have indicated the importance of the adaptive immune system to the development of atherosclerosis. Hypercholesterolaemic Apo-E knockout mice produce significantly fewer atherosclerotic lesions if crossed with immunodeficient *scid/scid* mice([144]). The tendency to atheroma formation could be re-instigated by the transfer of $CD4^+$ T cells from atherosclerotic donors to deficient mice.

These changes with inflammation could be mediated by a number of different mechanisms. Infectious agents may increase atherosclerosis directly, by initiating an inflammatory response in the vessel wall, or indirectly either by a generalised immune response or by inducing autoimmunity against cell wall components. That autoimmune cross-reactivity contributes to the inflammatory state is

supported by the observation that immunisation of mice with endogenous β2 glycoprotein-I (a ligand recognised by anti-phospholipid antibodies)([145]) or heat shock protein 60/65 led to increased atherosclerosis([146]). Similarly normocholesterolaemic rabbits have increased atheroma formation when immunised with heat shock protein 60 or Mycobacterium tuberculosis, which is rich in this antigen, and heat shock protein-60 expression is detectable within the plaques([147;148]).

## 1.6.2 Human data

### 1.6.2.1 Acute infections and cardiovascular disease

There is evidence for an increased incidence of acute coronary events following acute infections. This could occur through an alteration in the properties of inflammatory cells within atherosclerotic plaques to favour rupture, by the induction of endothelial dysfunction (which is discussed further in section 1.9) or by changes in the coagulation system to favour thrombosis.

Short-term increases in the risk of atherothrombotic disease have been seen following a wide range of inflammatory insults including respiratory infections, sepsis and surgery([149-151]). Patients presenting with acute coronary syndromes have raised inflammatory cytokines on admission and their level predicts outcome([152]). That this is not true for individuals with variant angina suggests that ischaemia *per se* does not increase cytokines.

Atherectomy samples taken during percutaneous coronary interventions support a role for acute inflammation. Meuwissen et al showed a correlation between the

presence of excess macrophages and T lymphocytes and the risk of future episodes of unstable angina([153]). These histological changes favour plaque rupture and consequent thrombosis. In children, acute infections have been shown to increase the presence of oxidised LDL and to be associated with a greater carotid intima medial thickness on ultrasound three months later([154]).

Cytokines released in response to acute infection induce an acute phase response that favours thrombosis by increasing fibrinogen, PAI-1 and tissue factor expression and increasing platelet expression of CD40. Fibrinogen concentration is itself predictive of future cardiovascular events([155]). *Ex vivo* experiments with human monocytes have shown that they activate the clotting cascade more readily, largely through tissue factor expression, when infected with *C. pneumoniae* or cytomegalovirus (CMV)([156]).

The incidence of acute myocardial infarction and strokes increases in the winter months, co-incident with an increase in the incidence of influenza infections ([157]). However, whether vaccination to reduce the incidence of influenza prevents events is not clear, as trials have found contradictory effects([158;159]).

### 1.6.2.2 Chronic infections and cardiovascular disease

In children under 15 years Pesonen et al showed, in a necropsy study, a correlation between infectious disease at the time of death and intimal thickening, which could be a precursor to atherosclerosis([160]). Serological studies have suggested that numerous agents, including *H.pylori*, *C. pneumoniae*, CMV and *herpes simplex*, may be involved in the inflammatory process that leads to

atherosclerosis([161;162]). In large studies there is a weak association between IgG antibodies against *H. pylori* and future cardiac events and a non-significant trend in favour of *C.pneumoniae*([163]), although in a later metaanalysis Danesh et al could not demonstrate a relationship with *H. pylori*([164]).

Associations shown by serological data are weakened by the common nature of these infections and the questionable importance of acute serological changes to chronic infective processes. It has been suggested that IgA levels are more relevant, as they may reflect ongoing infection, and these have been associated with acute coronary events. In addition, the lack of changes in antibody expression may mask an underlying cell mediated immune response that is more relevant to the development of atherosclerosis.

Local infection of atherosclerotic plaques has been shown, with varying degrees of certainty, for herpes viruses([162]), *C. pneumoniae*([165]), *H. pylori*([166]) and dental pathogens([167]) by a combination of electron microscopy, polymerase chain reaction, *in situ* hybridisation and directly from cultures. These data are also somewhat inconsistent as agents may only be detectable by one technique and in some cases are found in normal as well as diseased portions of arteries.

If infections are relevant to the development of cardiovascular disease they may allow the reduction in the risk of disease by treatment with readily available antibiotics. Given the relative preponderance of data for *C pneumoniae*, and to a lesser extent *H. pylori*, antibiotics against these agents have been investigated. Parchure and coworkers have shown that 5 weeks of treatment with azithromycin

leads to an improvement in endothelial function compared to placebo([168]). Antibiotic trials using clinical endpoints have been less clear. A secondary prevention trial of azithromycin (AZACS) failed to show any reduction in cardiovascular events after a short course of antibiotics([169]). The ROXIS study, using roxithromycin, only showed a significant effect in secondary prevention for the combined endpoint of death, myocardial infarction and recurrent ischaemia([170]). In a further secondary prevention study using roxithromycin even the secondary combined endpoint did not achieve significance, although this study was under-powered due to poor recruitment([171]). In the ACADEMIC trial, of azithromycin, the authors showed a reduction in inflammatory markers without any change in the clinical outcome in the 2-year follow-up([172]). Using clarithromycin as the treatment, the CLARIFY study showed a trend towards a reduction in cardiovascular events and a significant fall in the secondary endpoint of cardiovascular events and stroke([173]). It has been pointed out by a number of authors that in order to gain effective clearance a much longer duration of antibiotic treatment is required([174]). Larger studies with greater durations of antibiotics and longer follow-up periods are at present underway that will hopefully eliminate these areas of uncertainty.

A body of data has linked chronic periodontal infections with an increased risk of cardiovascular disease([175]), though this needs to be treated with caution to ensure it is not confounded by social class. In some studies there appears to be a correlation between the severity of the dental disease, in terms of alveolar bone loss, and the degree of coronary atherosclerosis([176]). This has been supported by animal data showing that oral inoculation of the periodontal pathogen

*Porphyromonas gingivalis* is associated with accelerated atherosclerosis([177]). Although the mechanism of this deleterious effect is not clear, studies in humans have shown an association between chronic severe dental disease, an inflammatory state and impaired endothelial function([178]).

The contradictory nature of serological and antibiotic intervention studies and their individual lack of power, has lead to the development of the concept of the "pathogen burden", the presence of a combination of multiple viruses and bacteria, as a cardiovascular risk factor([179]).

This can be further refined by considering the response of the host to infections and thereby their propensity to lead to atherosclerosis. Responses could in part be determined by the sex of the host([180]) or modified by genetic make-up. Polymorphisms of the CD14-monocyte-receptor gene promoter (the receptor for bacterial lipopolysaccharide), the tumour necrosis factor-α (TNFα) gene and the interleukin-1 gene have all been associated with an increased development of coronary heart disease in the presence of inflammatory stimuli([181]).

### 1.6.2.3 Chronic inflammatory conditions and cardiovascular disease

In addition to low-grade chronic infections as a stimulus for atherosclerosis it has been suggested that non-infectious inflammatory processes could also predispose to cardiovascular disease. Autoimmune diseases produce a similar inflammatory environment to infections, with raised cytokines, acute phase proteins, and cellular adhesion molecules and activated inflammatory cells.

Epidemiological studies have shown an excess of cardiovascular disease in individuals with systemic lupus erythematosis (SLE) (up to fifty-fold) and rheumatoid arthritis that cannot be explained by other "classical" risk factors ([182;183]). In SLE an association has been noted between the presence of TNFα and its soluble receptors (sTNFR1 and 2) and cardiovascular disease([184]). Treatment with TNFα antibodies has been shown to reduce disease activity in rheumatoid arthritis and also to improve endothelial function in these patients([185]). Effects of anti-TNFα antibodies on cardiovascular outcome have not been assessed. SLE may also increase the risk of atherosclerosis by mechanisms other than a generalised induction of an inflammatory response. For example SLE leads to the generation of autoantibodies against oxidised LDL([186]), which, in animal models, have been shown to combine with anti-cardiolipin β2-glycoprotein-1 antibodies and favour plaque formation([187]). Whether the increased prevelance of the antibodies to oxidised LDL are themselves pathogenic or reflect an increased concentration of oxidised lipids in this condition, is not yet clear. SLE has also been shown to be associated with markers of increased oxidant stress, which may also explain the increased oxidised lipids and consequent atherosclerosis([188]). Later in this section the association between C-reactive protein (CRP) values and cardiovascular risk will be explored (section 1.8.1.1). Interestingly, although SLE is correlated with an increased cardiovascular risk it is also associated with polymorphisms of the CRP gene, which lead to reduced basal and stimulated serum concentrations([189]).

In giant cell arteritis, an inflammatory condition associated with ischaemic cerebral events, a reduced concentration of the inflammatory cytokine interleukin-

6 (IL-6) was associated with increased adverse events([190]). It was suggested in this paper that the lack of IL-6 retards the development of neo-vascularisation that may protect from ischaemic events. Therefore although it is clear that chronic inflammatory conditions are associated with increased atherosclerosis, it is not yet apparent whether this is a consequence of the inflammation or a more direct autoimmune phenomenon.

### 1.6.2.4 Inflammatory markers and cardiovascular disease

In humans, elevated concentrations of circulating markers, even in the pre-clinical phase of disease, suggest that inflammation is important to pathogenesis. Observational studies have shown a correlation between raised levels of CRP, IL-6, intracellular adhesion molecule-1, serum amyloid A, E-selectin, P-selectin, TNFα, fibrinogen, vWF, PAI-1, homocysteine or prior infection with common pathogens, and atherosclerosis and its complications([191-195]). This will be covered in more detail in section 1.8.1.1.

### 1.6.2.5 Atherosclerosis itself as an inflammatory stimulus

Several lines of evidence suggest that atherosclerosis is not only influenced by systemic inflammation, but that pro-inflammatory mechanisms occur locally in lesions. Histological data shows the presence of inflammatory cells within atherosclerotic plaques, primarily macrophages early on in the process and then increasingly neutrophils during periods of instability([196]).

Activated macrophages within atherosclerotic plaques have been shown to increase the expression of pro-inflammatory cytokines such as TNFα, IL-1β, IL-8, MCP-1 and membrane expressed type 3 matrix metalloproteinase (MMP)([197;198]). Activated endothelial cells overlying atherosclerotic plaques express adhesion molecules (VCAM-1, ICAM-1 and selectins) in the same way as activated endothelial cells in post-capillary venules. These mediators could then play a role in developing and destabilising lesions within the vasculature.

Generalised inflammation within the coronary tree has been shown in individuals with acute coronary syndromes by the presence of activated neutrophils not just at the site of the "unstable" plaque([199]). The presence of inflammation within plaques has also been demonstrated by both [$^{18}$F]-flurodeoxyglucose positron emission tomography scans and assessment of the local temperature of plaques and been shown to be correlated with the presence of increased numbers of inflammatory cells and a detrimental clinical outcome([200;201]). As such the association between inflammatory markers and outcome may reflect a response to unstable plaques, rather than a response of plaques to inflammatory markers.

#### 1.6.2.6 Effects of cardiovascular therapies on the indices of the inflammatory response

A number of therapies have been shown to significantly improve outcome in cardiovascular disease. Most dramatically acetylsalicylic acid (aspirin) has been shown to reduce atherothrombotic events in the coronary, cerebral and peripheral circulations in both the short and long term([202]). This effect of aspirin is generally thought to be largely due to its inhibition of prostanoid production by platelets,

which is anti-aggregatory, although anti-inflammatory effects cannot be discounted. Aspirin is known to reduce levels of inflammatory marker([203]). In support of an anti-inflammatory role, the protective effect of aspirin appeared greater in those individuals with an elevated baseline CRP value in one epidemiological study([204]).

Anti-inflammatory mechanisms have been proposed as a mechanism of action of HMG-CoA reductase inhibitors (statins) and angiotensin converting enzyme inhibitors (ACEi) in addition to their effects on lipid profiles([205]) and renin-angiotensin system([206]) respectively.

Studies of human carotid atherectomy specimens have shown a change in plaque morphology after 3 months of statin treatment to one containing greater amounts of collagen and a reduction in inflammatory cells and MMP-2 expression, which is typical of plaques at low risk of rupture([207]). Whether this apparent anti-inflammatory effect is due to reduction in oxidised lipid locally (over and above that seen systemically) or an as yet undefined alternative action of statins is not apparent. Likewise ACEi could be potentially anti-inflammatory by their effects on bradykinin concentrations([208]) or through the inhibition of angiotensin-II induced IL-6 production([209]). However, whether these are important effects *in vivo* over and above the known alteration in blood pressure induced by these agents is not clear.

In the same way, favourable effects on the level of inflammatory markers have been reported for both peroxisome proliferator-activated receptor (PPAR)-α and

PPAR-γ agents, in particular their ability to lower CRP concentrations([210;211]). These effects may represent direct changes by reduction in IL-1([212]), or rather reflect the positive effects of increased high-density lipoprotein and improved glycaemic control.

## 1.7 Focus of this thesis

This introduction indicates the importance of inflammation and endothelial dysfunction in the development and progression of atherosclerosis. The remainder of this chapter will focus upon the two areas of particular interest that form the background to the experiments described in this thesis.

Firstly it will explore the present understanding of the relationship between inflammatory markers and cardiovascular disease, with a very strong emphasis on C-reactive protein. Secondly there will be a discussion of the phenomena of inflammation-induced endothelial dysfunction.

These areas will be discussed in detail, as the thesis will then go on to describe a set of experiments that attempt to determine the direct vascular effects of CRP both *in vitro* and *in vivo* and further work looking at the mechanisms of inflammation-induced endothelial dysfunction.

## 1.8 Inflammatory markers and cardiovascular disease

### 1.8.1 Observational studies of inflammatory products and cardiovascular disease

If sub-clinical inflammation is important in atherosclerosis, concentrations of inflammatory factors should be associated with the risk of future cardiovascular disease and their measurement might serve as a useful predictive tool. Given the proposition that "traditional" risk factors incompletely predict the occurrence of cardiovascular disease at both a population and individual level a great deal of interest has been focused on so called "novel" risk factors. Some of these are other biochemical abnormalities with a basis in basic research, such as homocysteine, whereas others are sub-fractions of existing parameters, like lipoprotein a (Lpa). This section will discuss the association of inflammatory markers and the risk of future cardiovascular disease.

#### 1.8.1.1 C-reactive protein

There has recently been an enormous interest in using CRP concentration as a tool to predict clinical atherosclerotic events. This has largely been fuelled by the development of high sensitivity assays that allow the reliable quantification of CRP concentrations within the range of values observed in overtly healthy adults (less than 5mg/L). Individual differences in the concentration of CRP in this range can stratify risk in individuals without evident cardiovascular disease.

In initially healthy male individuals, enrolled in the MONICA study, a raised baseline CRP concentration was associated with an increased future risk of an atherothrombotic event over a follow-up period of 8 years (hazard ratio 1.5, 95% confidence interval 1.14-1.97)([213]). A similar result was seen in the Physician's Health Study where healthy men were followed up for over 8 years and CRP concentration at baseline again predicted the frequency of atherothrombotic events (odds ratio 2.9 comparing lowest to highest quintiles of CRP [0.1-0.7mg/L vs. 3.8-15mg/L])([204]). Likewise baseline CRP concentrations were found to predict the development of peripheral vascular disease([214]). The Women's Health Study also showed that a raised basal CRP value, in apparently healthy women, was predictive of atherothrombotic events during the 3 year follow-up of this study (relative risk 4.8 for same groups as described in Physicians Health Study)([194]). In a further healthy population, women on average have a higher CRP concentration than men (but lower cardiovascular risk), and this is further increased if they are on hormone replacement therapy. It is not clear, however, whether this increase in CRP is correlated with an increased risk or whether the baseline value for normality is different in men and women([215]).

In the setting of an acute coronary syndrome CRP has been shown in a number of studies to predict future risk of recurrent events. In the FRISC study, a randomised controlled trial of low molecular weight heparin in acute coronary syndromes, a CRP concentration of <2mg/L was associated with a 5.7% risk of a cardiovascular event during follow-up (mean 37 months), whereas a CRP>10mg/L carried a 16.5% risk ($P<0.01$)([216]). A European study showed a similar trend over a shorter follow-up period (mean 6 months)([217]). Similarly, the

degree of elevation of CRP following an acute myocardial infarction stratifies individuals in terms of the risk of death and their life expectancy([218]).

Coronary angioplasty for the treatment of obstructive atherosclerosis is known to carry a degree of risk according to the anatomy of the disease and the pre-morbid condition of the patient. CRP concentration, prior to the procedure, has been shown to predict an adverse event, myocardial infarction and death, in the following 30 days, after adjustment for other risk factors([219]). In a detailed study of the time course of changes in CRP concentration following angioplasty, a rise in inflammatory markers was only seen in those with unstable symptoms and a pre-existing increase in CRP value([220]), thereby pointing towards a group with increased tendency to develop a marked inflammatory response. Following percutaneous intervention, the rise in inflammatory markers, CRP, IL-6 and TNFα, is predictive of an adverse outcome, while intra-procedural glycoprotein IIb/IIIa inhibition damps this inflammatory response([221]). The ability of CRP to predict outcome of coronary artery bypass grafting surgery is less clear as some studies indicate a link and others do not([222;223]). CRP values may also predict the morphology of coronary plaques. In a study of patients presenting with an acute coronary syndrome intravascular ultrasonography demonstrated a correlation between a raised CRP (>3mg/L) and the presence of plaque rupture, though whether CRP was pathogenic or a marker could not be differentiated([224]).

It is important to place CRP values within the framework of existing predictors of cardiovascular risk. One study has suggested that CRP concentration is actually a superior predictor of cardiovascular disease than any other risk factor([225]), though

this has been challenged by a large prospective analysis of an Icelandic population([226]). Interestingly an inflammatory milieu has been shown to predict the development of Type 2 diabetes mellitus, itself a powerful risk factor for cardiovascular disease, though in this case only IL-6 concentrations were predictive when body habitus was controlled for([227]). Likewise a cross-sectional study and a prospective cohort study have suggested an association between CRP values and hypertension, indicating it may, in part, have an inflammatory aetiology([228;229]).

Not every correlation study has identified CRP as a predictor of adverse outcome. CRP concentrations do not correlate with the presence of coronary artery calcification, which is thought to represent existing disease and is itself a predictor of future complications([230]).

The stimulus leading to the increased basal levels of CRP is not clear. It may represent a response to a mild systemic inflammatory stimulus, the presence of atherosclerosis or reflect an underlying genetic tendency. Studies with monozygotic twins have shown a significantly higher correlation of baseline CRP concentrations compared with dizygotic twins, indicating an important genetic component to the control of basal production([231]). A number of genetic variations in both the CRP gene and the genes of the cytokines that stimulate its release have been described. A dinucleotide repeat polymorphism in the intron of the CRP gene has been shown to predict CRP values([232]), as has a polymorphism in the 3' untranslated region (+1444C/T)([233]). Further variants, which are associated with an increased antinuclear antibody production, are also predictive of lower basal

CRP concentrations([189]). Likewise polymorphisms in IL-1 and IL-6 can alter the basal and stimulated concentrations of these cytokines, and thereby change CRP values([234;235]). The importance of these genetic factors in the use of CRP as a predictive marker is not yet fully determined([236]).

Most of the studies described rely on a single measure of CRP at baseline. This has been questioned by a reanalysis of the MONICA database, which shows an intra-subject variation in CRP concentrations on re-measurement after 3 years, with a reliability coefficient of only 0.54. The authors suggest that CRP ought to be repeated three times before confirming a baseline level([237]). A much smaller degree of variation was found when CRP was reassessed over a short period, such as a few days. Similarly, in the Icelandic population, where remeasurement occurred after 12 years, they found a correlation of 0.59([226]).

### 1.8.1.2 Interleukins

Similar studies looking at the prognostic implications of raised inflammatory cytokines have been performed, though these suffer from the more dynamic nature of these markers in response to intercurrent infections and circadian rhythms.

Increased concentrations of IL-6 have been shown to be predictive of future cardiovascular events in a prospective study of nearly 15000 apparently healthy men([238]). In individuals with angiographically documented coronary artery disease, IL-6 and IL-1β predicted the presence of ischaemia on 48 hour Holter monitoring (IL-6 concentration 3.9pg/ml for cases vs. 1.7pg/ml for controls) and

IL-1β predicted multi-vessel disease([203]). Unlike CRP, concentrations of IL-6 are predictive of the presence of diffuse atherosclerosis, both in the form of three-vessel coronary artery disease and peripheral vascular disease([239]).

Following admission with unstable angina IL-6 and IL-1Ra levels predict the risk of in hospital complications, particularly with comparisons between a sustained cytokine response and a rapidly falling inflammatory level([152]). In the setting of unstable angina IL-1Ra has also been shown to discriminate between individuals with atheroma and stable and unstable symptoms, and to predict prognosis([240]).

Recurrent ischaemic cardiovascular events have been shown to be more common following an acute myocardial infarction in individuals with raised TNFα([241]). In a prospective study of cardiovascular events, in a group of subjects with angiographically documented coronary artery disease, IL-18 was shown to be predictive of an adverse outcome, even after controlling for other inflammatory markers([242]).

### 1.8.1.3 Other inflammatory markers

Fibrinogen, which is an acute phase protein, has been shown to predict the occurrence of cerebrovascular events and advanced atherosclerosis, though not plaques with a large lipid content which are thought to be more prone to rupture([243]). Another acute phase protein, serum amyloid A, has been shown to predict mortality in the setting of an acute coronary syndrome([244]).

Macrophage numbers are increased in unstable atherosclerotic plaques and serum monocyte chemoattractant protein-1 concentrations have been shown to predict future cardiovascular death following an acute coronary syndrome([245]).

In addition to inflammatory markers other soluble factors have been found to be predictive of cardiovascular disease and of endothelial dysfunction. In particular soluble adhesion markers have been correlated with future events in individuals with documented coronary artery disease, though only sVCAM-1 remained predictive once markers of inflammation were included in a regression analysis([246]). However, doubt has been cast as to whether the presence of raised soluble adhesion markers adds any information over that provided by other risk factors([247]). This is important mechanistically in terms of inflammation as VCAM-1 in critical to the adherence of inflammatory cells to the endothelium and thereby their margination and the commencement of changes within the vessel wall.

#### 1.8.1.4 Conclusion

As CRP has become pre-eminent due to its high degree of self-correlation over time, easy assay and strength of association sections 1.8.2 and 1.8.3 will discuss this protein further. The observed association of CRP concentrations with cardiovascular risk has driven a great deal of interest in the idea that CRP may be causal in atherosclerosis. This work is detailed in a later section (1.8.3), though it is important to state early on that an association does not indicate causality. All of these correlation studies rely on adequate control for confounding, for example by smoking and age, which are known to be pro-inflammatory. Even in those where

this has occurred this does not allow for unmeasured confounders, such as other inflammatory markers, and the possibility of reverse causality, as would occur if atherosclerosis itself raised CRP concentrations. To understand this better the next sections (1.8.2 and 1.8.3) will describe the physiology and pathophysiology of CRP.

## 1.8.2 Physiology of CRP

CRP was identified by its ability to bind the somatic C-polysaccharide of streptococcus pneumoniae and was the first acute phase reactant described([248]). The acute phase response consists of non-specific physiological and biochemical responses to most forms of tissue damage. This involves the synthesis of a number of largely hepatic proteins, under the control of cytokines generated at the site of injury. In healthy young adult blood donors the median circulating CRP concentration is 0.8mg/L ($90^{th}$ centile 3.0mg/L), though this can increase during an acute infection to more than 500mg/L([249]). In this section I will describe the structure, metabolism and proposed functions of CRP.

### 1.8.2.1 Structure of CRP

The human CRP molecule consists of five identical non-glycosylated polypeptide subunits each containing 204 amino acids with a total molecular weight of 115135 Daltons. The protomers are non-covalently linked in an annular configuration to form a cyclic pentamer([250]). As such CRP is one of two human calcium-dependent ligand-binding plasma protein pentraxins, the other being serum amyloid P component, from the Greek *penta* (five) *ragos* (berries). Each subunit

has a characteristic "lectin-fold" made up of a two-layered β sheet with flattened jellyroll topology. The ligand-binding site, on the concave surface, consists of loops with two calcium ions bound 4Å apart by protein side chains, while the convex face carries a single α helix([250]).

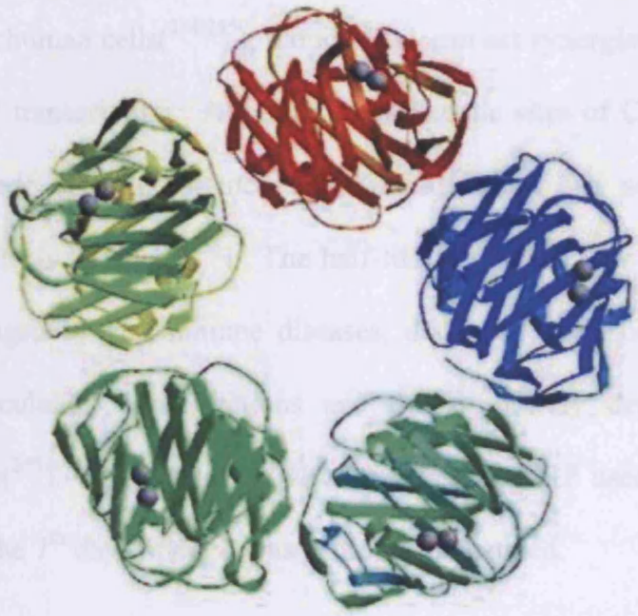


**Figure 1-3: Structure of C-reactive protein showing the pentameric structure and calcium ion binding sites([249]).**

The structure of CRP, in terms of sequence, subunit organisation and protein folding, is highly conserved with homologous proteins throughout the vertebrates and even in the arachnid Horseshoe crab (*Limulus polyphemus*). However there are variations with respect to ligand-binding specificity, the presence and distribution of glycosylation and protomer assembly([251;252]). As a consequence species differ in terms of the capacity of the CRP to precipitate and aggregate ligands, and to activate autologous complement proteins([253]).

### 1.8.2.2 Synthesis and Metabolism

Plasma CRP is produced by hepatocytes, predominantly under the control of IL-6, in response to "tissue injury". IL-1β or TNFα alone cannot increase CRP production in human cells([254;255]), though IL-1 can act synergistically with IL-6 to increase CRP transcription. Although extra-hepatic sites of CRP synthesis have been proposed, their importance and whether they can secrete as well as synthesise CRP is not clear([256]). The half-life of CRP is very stable at 19 hours, being unchanged by autoimmune diseases, diabetes, sepsis or neoplasia and as such the circulating concentrations are almost entirely dependent upon the synthesis rate([257]). In this study 90% of the labelled CRP used was recovered in the urine by the 7$^{th}$ day, where it was excreted unchanged.

CRP is encoded by a single gene on chromosome 1 which consists of two exons with an intervening intron and 5' promoter and 3' untranslated region. Polymorphisms of this gene and the genes for the stimulatory cytokines were discussed in section 1.8.1.1. Two pathways have been found to be important in the transduction of the effect of IL-6. In response to IL-6 CCAAT/enhancer binding protein beta and delta bind to multiple sites on the CPR promoter, including a critical site at –53. IL-6 has also been shown to act through signal transducer and activator of transcription-3 (STAT-3), which binds at –108 in the CRP promoter. Whereas IL-1 activates first the Rel protein/NF-kB family of transcription factors, in particular Rel p50 protein (or in some cases p50/p65 heterodimers) that then recognises a non-consensus kappaB site at –46 in the

promoter([258]). Although IL-1β alone cannot increase CRP production, maximal stimulation is only achieved when both these overlapping sites are occupied. Over-expression of nuclear factor-kappaB (NF-κB) can lead to a similar synergism in the IL-6 stimulation of CRP production, perhaps at the classical NF-κB binding site at -2652([259]).

Shortly after synthesis, monomers of CRP are formed into the stable pentameric structure within the endoplasmic reticulum (ER). There is some evidence in rabbit models that CRP can be retained in the ER and its efficiency of secretion can be increased in the setting of an acute phase response([260]). It is not clear if this also occurs in humans and caution must be exercised when comparing species as basal CRP values vary and CRP does not behave as an acute phase protein in all species. For instance the basal circulating concentrations in the rat are high (300-600mg/L) and do not vary with acute injury([251]).

Although human CRP concentrations increase during illness they are reasonably stable over time (provided acute inflammatory episodes are discounted, the variation is similar to that seen with cholesterol), and in particular do not appear to possess diurnal variation([261]). This is despite a well recognised diurnal variability in IL-6 concentration([262]). This most likely represents a "damping" of the diurnal variation of IL-6 since a rise in CRP lags by approximately 6 hours after exogenously administering IL-6([263]).

CRP concentration is positively correlated with age, body mass index (BMI), smoking history, hypertension and diabetes and may vary with menstruation([213]). Smoking and obesity (BMI>30 vs <25kg/m$^2$) are each associated with a doubling of CRP value. Eating does not alter CRP values and there are no specific pharmacological inhibitors of production. Agents, such as statins, that reduce CRP most likely acting by altering the level of inflammatory stimulation([212]).

Some inflammatory disorders, notably systemic lupus erythematosus, scleroderma, ulcerative colitis and leukaemia, result in a disproportionately small rise in CRP. In these conditions, the ability to increase CRP concentration is maintained during infection and it is not clear at this stage why this marker does not adequately reflect the level of inflammation of the underlying condition([249]). It is however of interest, as although CRP does not rise with SLE, this condition is associated with a marked increase in cardiovascular disease, as discussed in section 1.6.2.3.

### 1.8.2.3 Function of CRP

Although it is clear that CRP forms part of the acute phase response it is less clear exactly what functions it performs. It has been shown to be able to recognise and preferentially bind to phosphocholine and oxidised lipoproteins in damaged cell membranes, though not native lipoproteins([264]). CRP is also able to recognise other autologous ligands, including phosphoethanolamine, small ribonucleoprotein particles and extrinsic ligands including many glycan and phospholipid constituents of microorganisms([265]). This specific binding repertoire

allows it to selectively attach to capsulated bacteria, fungi, parasites and apoptotic cells.

Human CRP bound to these macromolecular ligands, or aggregated, is recognised by C1q and can therefore activate the classical complement pathway. Through engaging the adhesion component C3 this results in the formation of the terminal membrane attack complex (C5-C9)([266]). There is some evidence that bound CRP is also able to activate the alternative complement pathway by binding factor H and activating C5 esterase([267]). There is conflicting data as to whether rat CRP is able to activate its autologous complement([251;268]). Activation of complement will lead to opsonisation and clearance of pathogens and necrotic host tissue. Given these observations it has been proposed that CRP forms part of the innate immune system, which provides initial protection prior to the upregulation of the acquired immunity.

Some authors have proposed that CRP possesses a low affinity Fcγ ligand that will allow it to opsonise cells directly and activate the immune system in the same way as immunoglobulins([269]). These observations have been questioned in terms of the quality of controls used and in particular the use of whole IgG anti-CRP antibodies in the studies. The Fcγ ligands on this IgG are of very high affinity and any contamination would swamp any proposed effect of CRP. In carefully controlled studies using recombinant highly purified CRP binding to Fcγ ligands could not be confirmed([270;271]).

Support for a protective role for CRP has been provided by studies with transgenic mice expressing rabbit CRP. In this model the animals are relatively protected from lethal endotoxaemia, when compared to litter mates not over expressing CRP([272]). Mice can also be protected from *Streptococcus pneumoniae* by either passive injection of human CRP([273]) or transgenic modification([274]). Importantly these effects require the presence of complement and do not appear to be mediated by the controversial Fcγ ligands, suggesting that to a large extent the protective effects of CRP are mediated by its ability to activate complement([275]). It is of note that CRP is not an acute phase protein in mice and the effects seen are thereby mediated by the heterologous human pentraxin.

A large number of other potentially pathological functions have been ascribed to CRP, which will be considered in the next section (1.8.3). Caution has to be applied to a large amount of this work as not all authors have rigorously controlled for potential contamination of substrates, especially with lipopolysaccharide and immunoglobulins, or accounted for the possible pitfalls of using heterologous CRP to the system being studied. Particular importance also has to be drawn to the near universal usage of commercially sourced CRP (from Calbiochem, Beeston, UK), which is produced for use as a standard to calibrate CRP assays and which is not necessarily completely free of protein contaminants and is supplied with a buffer containing sodium azide as an anti-bacterial agent. Previous work has shown the importance of sodium azide as a mitochondrial respiratory chain inhibitor and metabolic poison and as such it may interfere with any physiological process under investigation([276-278]). A group using rabbit vessels showed that CRP preserved in sodium azide caused acute vasorelaxation,

while CRP either made without sodium azide or in which the azide had been removed by dialysis showed no acute vasoactive effects([279]). Similarly another group has shown that the vasorelaxant effects of CRP in rat aortic rings are mediated by the azide([280]). Sodium azide is known to be a direct NO donor and this was thought to be the mechanism of this effect([277]).

### 1.8.3 Pathophysiology of CRP

A large number of studies have been performed looking at actions of CRP that are potentially harmful to the host. These are detailed in the sections below.

#### 1.8.3.1 Animal Studies

Using a rat model of myocardial infarction (ligation of the left anterior descending artery) the size of an infarct can be determined by nitroblue tetrazolium viability stains. Intra-peritoneal injection of human (azide free) CRP, but not its buffer, led to a 40% increase in infarct size([281]). This effect was prevented if the complement system of the animals was previously depleted with cobra venom, suggesting that complement fixation is required for this deleterious effect. Immunohistochemical staining located the human, but not rat, CRP to the infarct site, where it was co-localised with complement.

In a murine model of SLE, injection of chromatin leads to acceleration of the course of the disease, whereas when it is co-injected with human CRP, which is known to bind to chromatin, the animals did not suffer increased morbidity or mortality([282]). Similarly a transgenic model of mouse SLE which also over-

expresses human CRP showed a reduction in morbidity and mortality([283]). These results are used to support a role for CRP in preventing autoimmunity and immune complex clearance, though the relevance of co-injection or overexpression of an heterologous pentraxin into a mouse model of SLE, where CRP is not an acute phase protein, to the human condition is not clear.

The same group has studied the effects on vessel injury in human CRP over-expressing mice, in comparison to wild type, and shown a marked increase in femoral artery thrombosis (from 17 to 75% of animals)([284]). This correlated with a decrease in clotting time in these animals *ex vivo*. Whether these changes are a consequence of over-expression of tissue factor or other pro-coagulant proteins has not been explored.

Taken together these studies suggest potentially important effects of CRP. However given the concerns about interpreting studies performed in one animal with CRP from another animal (usually human) caution must be applied to their findings. As previously indicated, CRP although largely preserved between species does have different biological properties and dynamic responses and these may influence any results. The next section will describe studies in human models that therefore avoid this cross-species difficulty.

### 1.8.3.2 Human Studies

#### *1.8.3.2.1 Histopathological studies*

A number of authors have shown, with immunohistochemical techniques, that CRP can be found within atherosclerotic plaques and that it appears to co-localise

with complement([285;286]). It is not clear whether this is a primary effect or a reflection of the presence of oxidised LDL within these lesions. Intriguingly one group has shown the colocalisation of CRP in atherectomy samples with p22phox, a component of NADPH, although the functional importance of this observation is not clear particularly as other circulating proteins can also be found within atherosclerotic plaques([287]).

In addition to its localisation to atherosclerotic plaques CRP has been found in increased quantities in infarcted myocardial tissue. In areas of infarct of greater than 12 hours age, a post mortem study showed co-localisation of CRP with activated complement, suggesting a role for CRP in this situation([288]). This is of particular importance given the animal study described above using experimental infarcts, and supports a role for CRP in determination of myocardial viability post-infarct and a possible role in ischaemia-reperfusion injury.

#### *1.8.3.2.2 Cell culture studies*

Incubation of human aortic endothelial cells with CRP leads to an increase in the expression and activity of plasminogen activator inhibitor-1, which may favour atherothrombosis([289]). In the presence of serum, CRP (10mcg/mL) was shown to increase expression of VCAM-1, ICAM-1 and E-selectin by flow cytometry in human coronary artery cells and umbilical vein cells([290]). A similar model has also been used to show that CRP increases monocyte chemoattractant protein-1 and reduces NO production by venous endothelial cells and that this can be reversed with co-incubation with the PPARγ agonist rosiglitazone([291]). Human umbilical vein endothelial cells have also been shown to release endothelin-1 in

response to exposure to CRP, an effect that could be inhibited by blockade of IL-6 and endothelin receptors([292]).

Peripheral monocytes incubated with CRP appear to increase their expression of tissue factor, which has pro-coagulant properties and may favour atherothrombosis([293]). In follow on studies it has also been suggested that this effect can be potentiated by interferon-γ and lipopolysaccharide([294]). In addition to altering monocyte protein expression it has been suggested that CRP can induce migration of these cells([295]), though not all investigators have demonstrated this([296]).

In human vascular smooth muscle cells incubation with CRP has been demonstrated to increase angiotensin type-1 receptor mRNA and protein expression and, as a consequence, increase cell migration and reactive oxygen species generation *in vitro*([297]).

Of particular relevance to endothelial function two groups have studied the impact of incubating human aortic endothelial cells with commercial CRP and have shown a reduction in the expression of eNOS and reduced production of NO and cGMP([298;299]). In these experiments, the presence of contaminating lipopolysaccharide was excluded by the use of Detoxigel columns and specificity was explored by heat degradation of the CRP.

Taken together, these effects point towards CRP producing a marked prothrombotic and atherogenic phenotype within the endothelium and vasculature,

suggesting it performs a direct role in atherosclerosis rather than purely acting as a marker of underlying inflammation. As previously indicated caution must be applied to these conclusions as all studies used commercial CRP where concern has been expressed as to the importance of interactions with contaminants and the specificity of effects seen([249]).

#### *1.8.3.2.3 Ex vivo studies*

Very little experimental work has been performed demonstrating the effects of CRP on human tissues.

One group used sections of internal mammary arteries obtained at the time of bypass surgery to study contractile function of the vessels in organ baths. CRP, from a commercial source, caused a dose dependent relaxation of the vessel rings pre-contracted with endothelin-1, which was not dependent upon the presence of the endothelium or nitric oxide synthase activity. The effect appeared to be mediated by an alteration in potassium channel function as it was attenuated by pre-incubation with barium chloride that can inhibit inward rectifier potassium channels([300]). Surprisingly nearly 50% relaxation of the vessel occurred with $10^{-8}$M of CRP (equivalent to 0.2mg/L), which would suggest that "normal" concentrations of CRP would tonically relax these arteries *in vivo*. Caution must be applied to these findings in light of the studies indicating the direct vascular effects of CRP are mediated by sodium azide([279;280]).

*In vivo* correlation studies are considered in section 1.9.2.1, and also within the section (1.8.1.1) on CRP and epidemiology.

In summary there are multiple potential pathogenic mechanisms described for CRP, which could make it instrumental in the development of atherosclerosis and not merely a marker for other inflammatory markers with a causal role. It could increase atherothrombosis by stimulating changes in both the coagulation system and the phenotype of the endothelium and mediate greater myocardial injury by increasing complement dependent cell lysis. Not all of the studies described include detailed and rigorous controls and some lack biological plausibility. As a consequence there is still a need for more, rigorously performed, investigations into the potential functions of C-reactive protein. One of the main aims to this thesis was to explore the direct vascular effects of CRP, free from commercial contaminants.

## 1.9 Inflammation and Endothelial function

This introduction has discussed evidence that atherosclerosis is an inflammatory disease and that acute inflammation may be involved in the development of acute coronary syndromes, while chronic, low-grade inflammation could increase the risk of later cardiovascular events. It has also indicated that endothelial dysfunction is probably an early process in the development of atheroma and may be important for the destabilisation of established plaques. Particularly as it occurs with all orthodox cardiovascular risk factors, is a systemic phenomenon and predicts future adverse events. A number of studies provide evidence for a link between inflammation and impaired endothelial function, and these are described in the next section (1.9.1).

### 1.9.1 Animal studies

It has been known for some time that in a large number of animal models bacterial endotoxin induces changes in vascular responses, whether given *in vivo* or *ex vivo* ([301-305]). Depending on the model, endotoxin causes either widespread vascular changes or an impairment restricted only to endothelium-dependent mechanisms. It has been shown that endothelium-dependent changes are due to alterations in NO synthesis, with an increase in iNOS activity with consequent downregulation of eNOS function resulting in endothelial dysfunction([306]). The importance of increased iNOS activity in this process has been confirmed by the inability to induce endothelial dysfunction in iNOS knockout mice exposed to endotoxin([307]). These functional changes are combined with changes in both basal and stimulated nitric oxide release([304]).

In rats, intravenous infusion of the inflammatory cytokine TNFα, prior to sacrifice, also led to a marked reduction in relaxation of blood vessels to the endothelium-dependent vasodilator acetylcholine, in comparison to control animals, with no alteration in the response to a direct nitric oxide donor([308]). Similarly *in vitro* exposure of bovine pulmonary arteries to TNFα for at least 30 minutes led to impairment in receptor mediated endothelium-dependent relaxation([309]).

Using cultured bovine endothelial cells it has been shown that exposure to endotoxin causes a reduction in both basal and stimulated nitric oxide from

endothelial NOS, indicating the development of endothelial dysfunction([304]). In these experiments a mixture of IL1β and TNFα alone did not produce the same effect, however a separate group has shown that TNFα can reduce eNOS mRNA stability and thereby decrease eNOS expression in a bovine model([310]).

## 1.9.2 Human studies

### 1.9.2.1 Correlation studies

Using CRP as a marker of underlying inflammation, several groups have shown an association between a raised concentration of this protein and endothelial dysfunction. Fichtlscherer et al demonstrated that a raised CRP was associated with impaired responses of the forearm vasculature to endothelium-dependent vasodilatation with acetylcholine, in subjects with documented atherosclerosis, in a manner independent of classical cardiac risk factors([311]). Cleland et al expanded this by showing that, in healthy volunteers, the relationship between serum CRP concentrations and endothelial responses is dependent on nitric oxide as the response to L-NMMA is also correlated with CRP values([312]). In a further study examining flow-mediated dilatation (FMD) in patients with documented peripheral artery disease, there was an association between raised CRP and fibrinogen and a relative reduction in FMD([313]). In children an association has been demonstrated between a raised CRP value and impaired FMD and increased intima medial thickness, though this was a cross-sectional study and did not look at future atherosclerotic disease([314]). In one study CRP, but not erythrocyte sedimentation rate (ESR), was correlated with endothelial dysfunction, shown by FMD, and then only in individuals with established atherosclerosis([315]).

In the setting of unstable angina one group has shown a correlation between the presence of a raised CRP concentration (>5mg/L) and increased vasoconstriction in a cold pressor test and increased vasodilatation to GTN at the point of the culprit intracoronary lesion([316]). Whether this is directly related to a change in endothelial function is not clear, although in other studies a correlation has been found between increased vasoconstriction to acetylcholine and the cold pressor test in human coronary arteries([317]).

These studies therefore show an association between an inflammatory phenotype and endothelial dysfunction though do not explore the specific mechanism or allow inference about a causal role for CRP or other inflammatory products. Other circulating inflammatory markers or other cardiovascular risk factors with which it is closely correlated may confound a correlation between CRP and endothelial dysfunction. This is highlighted by a similar study using FMD in subjects with familial hypercholesterolaemia and without clinical atherosclerosis, which found no association between CRP or selectins and endothelial dysfunction([318]). In this study no change in FMD was found with statin treatment, in contrast to a number of other studies described previously.

Evidence also exists that the presence of underlying infections is correlated with impaired endothelium-dependent responses. In a mixed diabetic and non-diabetic population seropositivity for CMV, but not *H. pylori* or *C. pneumoniae*, was associated with impaired vasodilatation to bradykinin([319]). In contrast to these positive studies Khairy *et al* failed to show an association between endothelial

dysfunction, assessed with FMD, and the presence of antibodies to C. pneumoniae, CMV, EBV or H. pylori, or to CRP concentrations, in healthy young male volunteers([320]). As indicated before, advanced periodontal disease has been shown to be associated with an elevation in CRP and a reduction in FMD, relative to healthy controls([178]). Again these studies are unable to determine the mechanism by which these effects occur, or whether they are causal or simply associative.

### 1.9.2.2 Hand vein studies

The dorsal hand vein provides a relatively accessible vessel to study *in vivo*, as it can be isolated from the circulation and agents selectively instilled and the responses studied. Impairment in the response to noradrenaline in these vessels has been shown following the instillation of pro-inflammatory endotoxin, indicating an increase in NO production([321]). In this case the effects were not reversible with either inhibitors of nitric oxide or cyclo-oxygenase synthesis, though they were preventable with hydrocortisone. In the same model the cytokines ILβ and TNFα reduced vasodilatation to the endothelium-dependent agent bradykinin, with no effect on endothelium-independent vasodilators. Endothelial dysfunction was prevented by both hydrocortisone and aspirin. The effect for each cytokine was of short duration; but could be prolonged by their combination([322]). This work suggests that the endothelial dysfunction seen following an inflammatory insult can be modelled *in vivo* in humans and may be relevant to cardiovascular disease. As indicated in an earlier section, this model was also used to demonstrate that IL-1β induced the up-regulation of $BH_4$

synthesis, and that the balance of this and increased co-factor requirements may be important in the development of endothelial dysfunction([323]).

### 1.9.2.3 Arterial studies

Arterial studies using a combination of venous occlusion plethysmography and FMD have been performed *in vivo* in humans using two inflammatory models – typhoid vaccination and intravenous endotoxin administration.

Our group has developed a model of acute inflammation, using typhoid vaccination, which allows the study of changes in vascular reactivity in the period before and shortly after the stimulus. It has been shown that 8 hours following vaccination there is a marked reduction in vasodilatation in the forearm in response to the endothelium-dependent vasodilators acetylcholine and bradykinin. There is no change in the response to endothelium-independent vasodilators and all effects have normalised by 32 hours. A similar reduction in endothelial function has also been shown in response to a flow stimulus([324]). These changes are accompanied by an inflammatory cytokine response and it has been separately shown that an anti-inflammatory dose of aspirin prior to vaccination can prevent the endothelial dysfunction and modify the cytokine response. No improvement was seen with the use of local aspirin 8 hours post vaccination([50]). These studies show that acute inflammation, which may be prostanoid driven, causes endothelial dysfunction; however local dysregulation of prostanoid pathways do not account for the endothelial function that has been observed. At the time the work for this thesis was undertaken it was not clear whether the changes are due to alterations in NO, EDHF or vasoconstrictor agents, or how inflammation leads to these changes.

Another group has shown somewhat contradictory results, where typhoid vaccination did not alter the response to bradykinin or acetylcholine although it did cause increased release of tissue plasminogen activator([325]). The failure to demonstrate endothelial dysfunction in these studies may be the result of different methodology, particularly the use of higher doses of vasodilators that may have been beyond the linear portion of their response curves and the use of a shorter (6 hour) time point.

Acute administration of endotoxin intravenously (1-2ng/kg) to healthy volunteers has been shown to induce a hyporeactivity to adrenoceptor agents and the endothelial dependent vasodilator acetylcholine at 4 hours, by a mechanism not thought to involve changes in iNOS expression([326]). The same group has gone on to show that administration of high doses of ascorbic acid can restore the impaired response to acetylcholine to normal, though the mechanism of this effect is not explained([327]).

Taken together these various studies suggest that inflammation can induce endothelial dysfunction and that this may represent an important mechanism in the development and progression of atherosclerotic disease. The published data thus far does not clearly demonstrate the mechanisms involved in inflammation-induced endothelial dysfunction and more information is needed on the relevant inflammatory products involved. The last section of this introduction will explore potential pathways through which endothelial dysfunction can occur, and the

second half of the thesis will describe the experiments performed in an attempt to address these in this vaccine model of inflammation *in vivo*.

## 1.10 Mechanisms of Endothelial Dysfunction

In theory endothelial dysfunction may be caused by a deficiency in vasodilators, or increased vasoconstrictor activity. Available evidence indicates a predominant role for reduced activity of the NO pathway in experimental and human atherosclerosis.

### 1.10.1 L-arginine-NO pathway

Animal models of atherosclerosis show reduced endothelial nitric oxide synthase activity and nitric oxide synthase inhibition leads to accelerated atherosclerosis([328;329]). Human hypertension is associated with reduced whole body NO production([330]) and reduced vasoconstriction response to NOS inhibition with $N^G$-monomethyl-L-arginine([116]). These changes in nitric oxide bioavailability could reflect either decreased NO production or increased breakdown.

#### 1.10.1.1 Reduced NO production

Production of NO can be reduced by a number of mechanisms. There may be a lack of substrate for NOS, a deficiency in essential co-factors for the enzyme or a reduction in the quantity of the protein.

#### *1.10.1.1.1 Substrate deficiency*

The substrate for eNOS is L-arginine and as endothelial cell and circulating concentrations are several-fold higher than the Michaelis constant ($K_m$) of the enzyme, direct deficiency seems at first an unlikely explanation for reduced NO bioactivity. However it has been shown in both animal and human models that supplementation with L-arginine can improve endothelial function. This could be explained by changes in the $K_m$ of the enzyme, the presence of a competitive inhibitor of NOS, compartmentalisation of the enzyme and substrate, or mechanisms that reduce arginine concentrations within the endothelium.

##### **1.10.1.1.1.1 Animal data**

In animal models of atherosclerotic risk factors, specifically hypercholesterolaemia, acute and chronic supplementation with oral L-arginine leads to an improvement in vascular function and a reduction in the progression of atheroma([331;332]). This paradoxical effect given the usual excess of substrate could be mediated by any of the mechanisms described above.

There are at least two endogenous methyl-substituted arginines that compete with arginine for the active site of NOS. $N^GN^G$-dimethyl-L-arginine (asymmetric dimethylarginine; ADMA) has been shown in cholesterol fed rabbits, a model of atherosclerosis, to be increased and to act as a competitor to L-arginine for nitric oxide production, which may make NOS substrate dependent in this setting([333;334]). Acute administration of ADMA or $N^G$-monomethyl-L-arginine (L-NMMA) in rats has been shown to raise blood pressure, at least in part through

an increase in peripheral vascular resistance([335]). Likewise in rat models of salt-sensitive hypertension increases in ADMA have been implicated in the tendency to increased blood pressure by antagonising basal NO production and in separate experiments L-arginine supplementation has been shown to reduce this form of hypertension([336;337]).

One possible explanation of this "arginine paradox" has been the demonstration, in porcine pulmonary artery endothelial cells, of compartmentalisation of NOS. Studies have shown co-localisation of eNOS and the arginine transporter (cationic amino acid transporter 1) in caveolae formed by the cytoplasmic membrane([338]). This may lead to preferential utilisation of extracellular L-arginine by eNOS meaning that extracellular (generally 50-200μM) rather than intracellular (usually >800μM) concentrations of arginine are relevant to the enzyme's efficiency. In bovine aortic endothelial cells this dependence on transport mechanisms has been demonstrated by the observation of a ten-fold higher $K_m$ for eNOS for L-arginine in intact cells to cell lysate([339]).

In addition to breakdown by NOS to form L-citrulline and NO, L-arginine can also be degraded by arginase to produce ornithine and urea. In rat aortic endothelial cells Buga et al have shown the presence of both constitutive enzyme (arginase-1) in large concentrations and a form inducible by lipopolysaccharide (arginase-2)([340]). Initially it was thought that these high arginase concentrations may metabolise sufficient L-arginine to make it rate limiting. These experiments, however, also indicated that lipopolysaccharide and cytokines also increase the production of $N^G$-hydroxy-L-arginine from iNOS that inhibits arginase-1 and

therefore prevents the limitation of supply of substrate for this high-output isoform. As such the importance of arginase in maintaining L-arginine levels or making NOS dependent upon its concentration is not clear.

#### 1.10.1.1.1.2 Human data

In humans with cardiovascular risk factors the reports on the effect of L-arginine supplementation are inconsistent. Some authors have shown an improvement in resistance and conduit vessel function in individuals with hypercholesterolaemia, hypertension, smoking and diabetes([106;115;341-343]). In contrast, other groups, including our own, have failed to show any increase in endothelium-dependent responses after supplementation with L-arginine in subjects with hypertension or diabetes([344;345]). No improvement in similar experiments was seen in healthy volunteers without cardiovascular risk factors([346;347]).

Like the animal models, there are a number of possible explanations for this apparent paradox. In human studies an association has been shown between increased serum ADMA levels and the presence of some cardiovascular risk factors and markers of atherosclerosis([348-350]). In particular the study by Boger et al showed that ADMA is raised in asymptomatic hypercholesterolaemics and is associated with impaired vascular reactivity that is reversible with systemic L-arginine([349]). Infusion of ADMA into the forearm of healthy volunteers leads to reduced blood flow([351]), whereas systemic doses increase blood pressure([352;353]).

A rare disorder of amino acid transport, lysinuric protein intolerance, may indicate the presence of compartmentalisation of L-arginine and NOS in humans. In this condition there are low plasma levels of L-arginine and L-lysine and reduced NO

production, which can be improved with supplemental L-arginine([354]). Genetic studies have localised the defect to one of the cationic proteins involved in L-arginine transport and suggest that at least in this setting L-arginine transport may be rate limiting([355]).

There are only limited data to suggest a role for arginase activity in the regulation of L-arginine availability. In diabetic men, who are prone to erectile impotence that may be related to deficient NO availablity, increased expression of arginase II has been demonstrated with reduced NOS activity that can be improved by inhibition of the arginase pathway([356]). The importance of this pathway in atherosclerosis is not clear.

#### *1.10.1.1.2 Cofactor deficiency*

As described previously (section 1.4.1.1) NOS requires a number of co-factors to function efficiently, including tetrahydrobiopterin ($BH_4$)([31]). $BH_4$ availability is dependent upon the balance between its breakdown, by oxidation or utilisation by aromatic amino acid hydroxylases, and synthesis, which occurs locally by two pathways. In its reduced state $BH_4$ is biologically active as a cofactor for NOS. When NOS is deficient in $BH_4$ electron transfer by the enzyme is uncoupled and superoxide is synthesised rather than NO([357]). *De novo* production of $BH_4$ from guanosine triphosphate, which is thought to be the predominant synthetic pathway, occurs through a series of steps initiated by the rate-limiting enzyme, GTP cyclohydrolase-1 (GTPCH-1). In monocytes, once GTPCH-1 is upregulated, 6-pyruvol-tetrahydrobiopterin synthase can become rate-limiting, leading to an accumulation of neopterin that is therefore a reflection of monocyte

activation([358]). This does not occur in a mouse model of GTPCH-1 over-expression([359]) as, in other species, 6-pyruvol-tetrahydrobiopterin synthase does not become rate limiting and $BH_4$ is produced in excess. A salvage pathway also exists, using sepiapterin as a substrate, which does not involve GTPCH-1. When in excess, $BH_4$ acts through GTP cyclohydrolase-1 feedback regulatory protein (GFRP) to inhibit its own production by reducing GTPCH-1 activity.

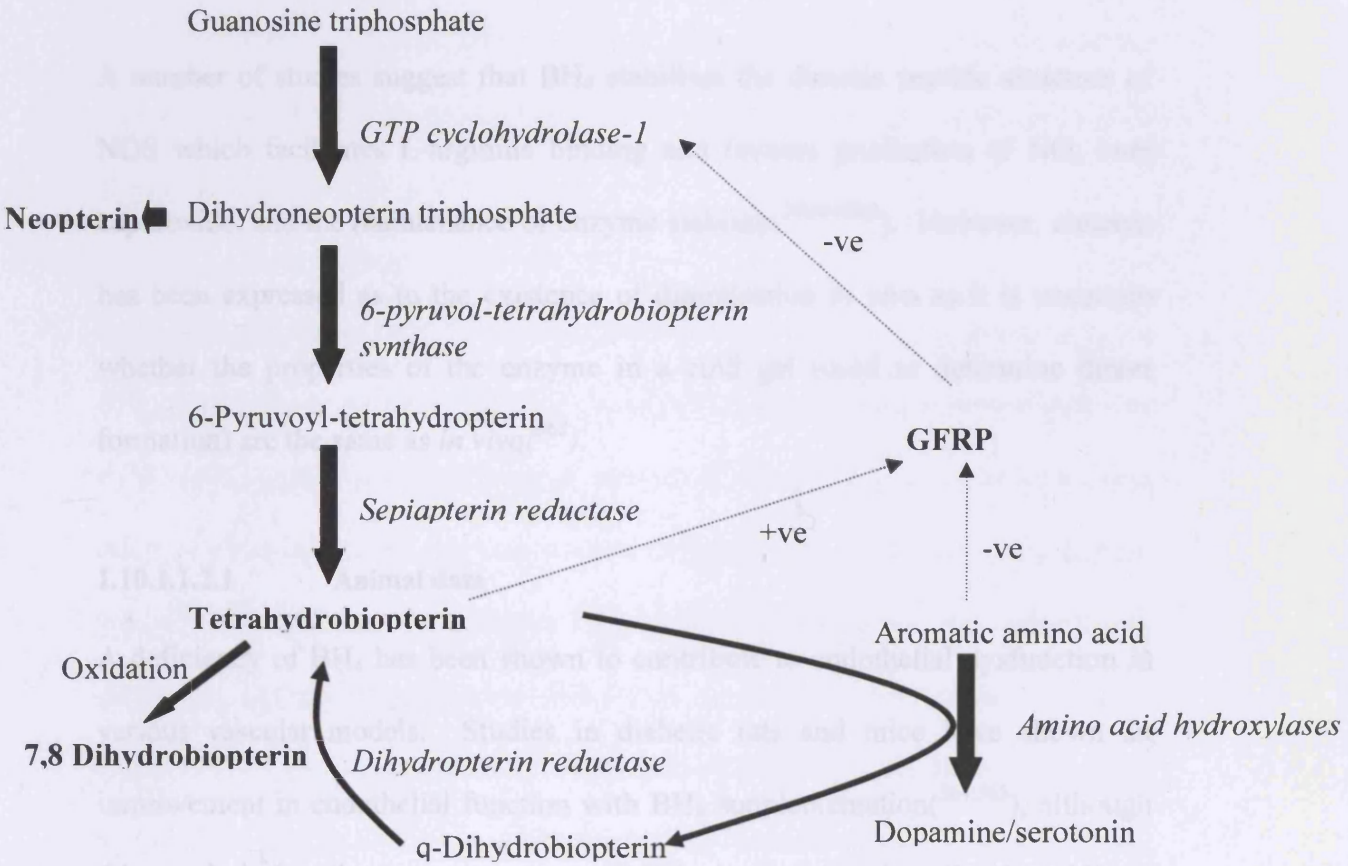



**Figure 1-4: Pterin pathway showing synthesis and potential routes of breakdown of $BH_4$.**

$BH_4$ is generated from GTP in a three-stage process controlled by the rate-limiting enzyme GTPCH-1. $BH_4$ is then broken down by oxidation or amino acid hydrolyses. GTPCH-1 is controlled by inhibition with GFRP, which itself is

under the control of $BH_4$. ($BH_4$ = tetrahydrobiopterin, GTPCH-1 = GTP cyclohydrolase-1, GFRP = GTP cyclohydrolase-1 feedback regulatory protein)

The exact effect of $BH_4$ on NOS is not clear. As NOS bears similarities to monooxygenases, for which $BH_4$ is also a cofactor, it has been proposed that $BH_4$ aids hydroxylation of one of the amidine nitrogens of L-arginine; however there are no data to support the cycling of $BH_4$ concentration that would then be expected during NO production if it acted in this way([360]).

A number of studies suggest that $BH_4$ stabilises the dimeric peptide structure of NOS which facilitates L-arginine binding and favours production of NO, over superoxide, and the maintenance of enzyme stability([30;361;362]). However, concern has been expressed as to the existence of dimerisation *in vivo* as it is uncertain whether the properties of the enzyme in a cold gel (used to determine dimer formation) are the same as *in vivo([363])*.

#### 1.10.1.1.2.1 Animal data

A deficiency of $BH_4$ has been shown to contribute to endothelial dysfunction in various vascular models. Studies in diabetic rats and mice have shown an improvement in endothelial function with $BH_4$ supplementation([364;365]), although this work lacks direct measurement of $BH_4$ in the vessel wall. In addition endothelial dysfunction of spontaneously diabetic rats has been shown to be mediated by a deficit in GTPCH-1 activity leading to reduced $BH_4$([366]), rather than by a reduction in eNOS protein. In these studies other NOS cofactors, calmodulin and calcium, were not different between control and diabetic animals, with NADPH and arginine actually being more abundant in diabetic cells.

Hypercholesterolaemic rabbits have been shown to have reduced quantities of $BH_4$ in vessel walls, with a relative increase in $BH_2$. Addition of sepiapterin increased the levels of $BH_4$ only in hypercholesterolaemic rabbits although this did not translate into improvements in vascular NO-dependent responses([367]), perhaps due to co-existent increases in $BH_2$ that may compete with $BH_4$ at the eNOS binding site. In an isolated vessel porcine model of ischaemic vascular injury, addition of sepiapterin or, to a lesser extent, methyltetrahydropterin improved previously impaired endothelium-dependent responses([368]). Similar experiments with pigs with atherosclerosis, following 4 months of a cholesterol-rich diet, also showed an improvement in the response to serotonin and substance P with sepiapterin([369]).

A number of theories have been put forward to explain this apparent deficiency in $BH_4$. An excess of oxidant free radicals, which are seen in hypercholesterolaemia ([370]), DOCA-sensitive hypertensive rats and mice([371]) and apoE knockout mice([372]), can inhibit $BH_4$ by a number of mechanisms. Firstly they may deplete NADPH or prevent the recycling of $BH_4$ by flavin nucleotides. Peroxynitrite, in particular, by oxidising $BH_4$ to $BH_2$, may uncouple the NOS in favour of superoxide production. Ascorbic acid (an anti-oxidant) has been shown in porcine endothelial cells to increase NO release by increasing $BH_4$ concentrations, presumably by favouring the reduced form of the pterin over $BH_2$, and thereby producing "coupled" NOS([373]). A reduction in generation of $BH_4$ would have the same effect as increased breakdown and there is some evidence, in rat macrophages, that oxidised low-density lipoprotein can prevent the normal up-regulation of GTPCH-1 by cytokine stimulation([374]).

GTPCH-1 expression, and thereby $BH_4$, can be increased by the same cytokines that increase NO production through iNOS induction([375]). Direct inhibition of GTPCH-1 in this model leads to a reduction in NO production. In addition, oestrogens have also been shown to increase GTPCH-1 expression([376]). In a murine diabetic model, GTPCH-1 over-expression led to increased $BH_4$ relative to $BH_2$ and consequent improvement in endothelial function in comparison to wild type diabetic animals([359]).

#### 1.10.1.1.2.2 Human data

In human studies relative $BH_4$ deficiency has also been implicated in endothelial dysfunction. In isolated vessels from individuals with known atherosclerosis on angiography, sepiapterin improved responses to acetylcholine and histamine with no effect on endothelium-independent vasodilators([369]). The same experiments showed no effect of sepiapterin in vessels from subjects undergoing valvular surgery and not known to have macroscopic atheroma.

Cell culture studies in endothelial cells also favour the paramount importance of adequate $BH_4$ for NO production([377]). As is evident *ex vivo*, adding ascorbic acid to human endothelial cells caused increased NO production in a $BH_4$-dependent manner([378;379]). As the control anti-oxidant (*Mn (III) tetrakis (4-benzoic acid) porphyrin chloride*; a superoxide scavenger) had no effect on NO production, and ascorbic acid had no effect in the presence of sepiapterin, it was proposed that in this model the ascorbic acid acted primarily through changes in $BH_4$ and not directly on NO. These effects are most likely by chemical stabilisation of the

pterin and reducing $BH_3^{\cdot}$ (the oxidative free radical of $BH_4$ produced by peroxynitrite([363])) to active $BH_4$. It has also been proposed that $BH_4$ partly acts to improve NO production by acting directly as an anti-oxidant; however in studies in smokers and hypertensive (animals) although $BH_4$ improved vascular reactivity an alternative reduced pterin with anti-oxidant properties (tetrahydroneopterin) did not([371;380]).

In human endothelial cells increased GTPCH-1 expression, using an adenoviral plasmid vector, leads to increased NO production in conjunction with an increase in NOS dimerisation([381]). Similarly, inflammatory cytokines in an *in vivo* human hand vein study have been shown to increase NO release by increasing $BH_4$ availability through excess GTPCH-1 expression([323]).

Infusion of $BH_4$ improves endothelial dysfunction in coronary vessels of patients with atherosclerosis *in vivo*([382]). In the presence of risk factors for atherosclerosis (hypercholesterolaemia([383]), smoking([380]) and diabetes([384])) $BH_4$ infusion improves endothelial function in the forearm, but has no effect in control individuals. In all these experiments the concentrations of $BH_4$ achieved are likely to be way in excess of the Km of eNOS for $BH_4$ and therefore non-specific effects cannot be ruled out.

Inadequate concentrations of $BH_4$ could represent a deficiency in production or excess breakdown. Smoking is thought to affect both mechanisms, with the aromatic amines of smoke inhibiting $BH_4$ production([385]) and the free radicals, particularly peroxynitrite, increasing its breakdown. Other cardiovascular risk

factors are also known to increase oxidant free radicals, as will be discussed below (section 1.10.1.2), and these may then lead to $BH_4$ deficiency.

#### *1.10.1.1.3 Reduced eNOS expression*

A reduction in expression of eNOS could limit NO availability. Animal work suggests that eNOS expression can be diminished by TNFα, hypoxia and a high LDL concentration([386]). Reduced expression of eNOS, accompanied by decreased NO production, has been shown in human vessels with established atherosclerosis, compared to normal arteries taken at surgery([387]). Changes in NOS expression, thought to be due to alterations in mRNA stability, have been observed in advanced atherosclerotic lesions, which show enhanced iNOS and diminished eNOS compared to normal sections of vessel([388]). The importance of these changes in the development or continuation of endothelial dysfunction is not clear.

#### *1.10.1.1.4 Alterations in NO signalling*

Endothelial dysfunction measured as an attenuation of agonist-mediated vasodilatation could also reflect an abnormality in agonist-mediated signal transduction. Nitric oxide release in response to signalling through G-protein coupled receptors is generally attenuated to a greater degree than that elicited by agonist independent increases in intracellular calcium mediated by a calcium ionophore (A23187) in both atheroscelerotic and post-transplantation models([389;390]). In a porcine model of hypercholesterolaemia, inhibition of G-proteins with pertussis toxin did not further impair endothelial function, in

contrast to control animals, suggesting that excess cholesterol had already altered the actions of these proteins([391]). Expression of G-proteins has shown to be reduced in tissue culture by exposure to oxidised LDL([392]) and in coronary arteries with age, hypertension and hypercholesterolaemia([393]). These changes could explain diminished responses to receptor-mediated agonists (such as serotonin) and also the variable impairment to different stimuli, though their importance in cardiovascular disease is as yet not clear.

### 1.10.1.2 Increased NO breakdown

Nitric oxide is rapidly oxidised in a number of steps culminating in the formation of nitrite and nitrate, and also by nitrosylating other molecules, particularly redox-activated thiols. As suggested previously (section 1.10.1), excess reactive oxygen species, especially peroxynitrite formed by reaction of superoxide and NO, may contribute to endothelial dysfunction and atherosclerosis. Free radicals can act directly to oxidise NO, reduce the bioavailability of $BH_4$ through oxidation or, as indicated above, alter G protein-coupled receptor function([392]).

Superoxide has emerged as an important free radical mediator and the sources of superoxide vary between vessels and species and include vascular NADPH oxidases([394]), present in endothelial and smooth muscle cells, "uncoupled" NOS([30;361;362;395]), xanthine oxidase([370]), and cytochrome p450. Mitochondria also generate superoxide as a bi-product of oxidative phosphorylation. Superoxide is metabolised by superoxide dismutase; the product, hydrogen peroxide, is subsequently metabolised by catalase to oxygen and water. Although superoxide reacts with NO three times more readily than with dismutase

($k_m$=6.7x10$^{10}$mol/L/s), in healthy vessels adequate tonic dismutase activity prevents NO breakdown, and oxidant stress only becomes pathological when superoxide production escalates or dismutases are inhibited([396]). Excessive oxygen free radicals may directly contribute to atherosclerosis by stimulating smooth muscle proliferation([397]), oxidising lipoproteins([398]) and favouring platelet aggregation and leukocyte adhesion([399]), in addition to reducing the bioavailability of NO.

#### *1.10.1.2.1 Animal data*

A number of animal models have shown the importance of changes in oxidant stress in the development of endothelial dysfunction and atherosclerosis.

In DOCA-sensitive hypertensive mice there is a reduction in NO bioavailability due to increased oxidant stress that is derived from eNOS. In knock-out models this has been shown to be initiated by superoxide, from NADPH oxidase, increasing $BH_2$ relative to $BH_4$ and thereby "uncoupling" NOS([371]). In the spontaneously hypertensive rat excess superoxide has been shown to be produced from xanthine oxidase, and inhibition of this enzyme with oxypurinol causes a fall in blood pressure([400]).

Hypercholesterolaemia is associated with endothelial dysfunction in part due to increased superoxide production, largely from xanthine oxidase, and thereby NO degradation([370]). Early atherosclerotic lesions in hypercholesterolaemic rabbits exhibit increased NADH oxidase activity, which can be reversed by angiotensin II inhibition, in keeping with the role of angiotensin II in the production of oxidant stress by the upregulation of angiotensin$_1$ ($AT_1$) receptors by lipids([401]).

Importantly, in this model, chronic angiotensin II inhibition led to the development of fewer atherosclerotic lesions. Similarly chronic administration of anti-oxidant vitamins can inhibit the development of atherosclerosis in a primate model([402]) and the development of endothelial dysfunction in cholesterol-fed rabbits([403]).

An alternative approach has been to supplement superoxide breakdown with polyethylene-glycolated superoxide dismutase, which has been shown in a rabbit model to partially correct impaired endothelial function([404]).

#### *1.10.1.2.2 Human data*

Excess NO breakdown in human vessels has been studied both *ex vivo,* by determining the presence of increased oxidant stress, and *in vivo*, with supplementation experiments.

In isolated human vessels the amount of superoxide produced by NADPH oxidases and the degree of endothelial dysfunction correlate with the number of cardiac risk factors([405]). Similarly increased levels of the components of NADPH, specifically p22phox, are seen in atherosclerotic vessels and vessels from individuals with diabetes([395;406]). Increased superoxide production from smooth muscle cells by NADPH oxidase occurs following stimulation by factors thought to be involved in atherosclerosis, including angiotensin II, thrombin, platelet-derived growth factor, tumour growth factor-α and lactosylceramide([407]). Studies in human coronary vessels suggest that there is a marked increase in xanthine oxidase and a decrease in extracellular superoxide dismutase in individuals with

atherosclerosis([396]). These assays were performed on the circulating enzyme after their release was evoked by heparin infusion, and therefore the importance of these observations to basal enzyme activity is not clear.

One manifestation of increased oxidant stress is the presence of oxidised phospholipids, in particular oxidised low-density lipoprotein (oxLDL). This has been localised within atherosclerotic plaques([408]) and been shown to be chemotactic to monocytes and scavenged by specific receptors([409]). OxLDL has been shown to promote an inflammatory state and atherosclerosis within endothelial cells by upregulation of growth factors and inflammatory proteins([410;411]). The susceptibility of LDL to oxidation (by measuring conjugated diene formation) is also correlated with the coronary vasomotor response to acetylcholine in hypercholesterolaemic individuals, even when considered independent of cholesterol lowering therapy([412]).

Increased oxidant stress in type 2 diabetes has been inferred from raised $F_{2\alpha}$ isoprostanes (stable eicosanoids formed by arachodonic acid oxidation), particularly following acute hyperglycaemia([413]), and $F_{2\alpha}$ isoprostanes have been found to be localised to atherosclerotic plaques in smokers([414]). A fall in total anti-oxidant status of plasma has been seen with the polycystic ovary syndrome, which is itself predictive of future cardiovascular disease([415]).

Ascorbic acid supplementation, that quenches superoxide, has been shown to improve endothelial function in the coronary and peripheral circulations in diabetics([416]), hypercholesterolaemics([417-419]), hypertensives([420]), smokers([421]) and

patients with chronic renal failure([422]). Whether these effects are mediated by an action of ascorbic acid to inactivate superoxide *in vivo,* despite the relatively low affinity of ascorbic acid ($k_m=10^5$M/s) compared to NO, through changes in $BH_4$([363]) or glutathione ([423]), remains to be determined. Ascorbic acid supplementation improved endothelial function in individuals with established coronary artery disease([424]) and the degree of improvement, which correlates with the degree of oxidant stress, is predictive of future cardiovascular complications([425]). Concern over the interpretation of the effects of ascorbic acid have been raised due to its ability to be pro-oxidant in the presence of iron, the "Undenfriend effect"([426]). In particular ascorbate does not protect against protein oxidation from stressors such as cigarette smoke([427]). It is not clear whether these effects are important *in vivo,* as there is little "free" iron present ([428]). Studies with vitamin E (α-tocopherol), another anti-oxidant vitamin that can be recycled by the anti-oxidant effects of ascorbate, have produced conflicting results with some studies showing enhanced vascular function([429;430]) and others indicating no positive effect([431;432]).

Consumption of the dietary antioxidant vitamin E and ascorbic acid, as part of a balanced diet and not by supplements, reduced cardiovascular events in prospective studies([433;434]). However randomised trials of anti-oxidant vitamin supplementation have produced conflicting results, with some showing reduced events([435]) but most others showing no improvement([436-438]). This may indicate that the prospective studies are confounded by a relationship between ascorbic acid intake and other cardiac risk factors, as has been suggested for social class in further studies from the EPIC-Norfolk group([439]).

Inhibition of superoxide production with the xanthine oxidase inhibitor oxypurinol has been shown to improve endothelial dysfunction of the forearm that occurs in hypercholesterolaemia, though it had no effect in individuals with hypertension([440]).

Oxygen free radicals are released in large quantities during sepsis, largely from the NADPH oxidase of neutrophils activated to clear the invading organism. Excessive oxidant stress in individuals with sepsis was predictive of organ dysfunction and impaired survival([441;442]). Endothelial dysfunction that occurs during sepsis may be mediated by iNOS induction and consequent eNOS down-regulation, though a role for increased oxidant stress, acting directly or indirectly upon the endothelium, has also been proposed([443]).

In summary there is a wealth of evidence suggesting that increased oxidant stress occurs in individuals with cardiovascular risk factors and this can lead to a reduction in NO bioavailability and endothelial dysfunction. The exact order of these events along with the relative importance of each component still needs to be determined.

### 1.10.2 Other pathways

Thromboxane is a potent vasoconstrictor, platelet-aggregating agent, and activator of adhesion molecule expression on monocytes. In animal models of atherosclerosis levels are increased. Inhibition of prostanoid synthesis, or selective thromboxane antagonists inhibit atherosclerosis([444]). In patients with

hypercholesterolaemia or those with established atherosclerosis, aspirin improves impaired endothelium-dependent dilatation([445;446]). These data suggest that the activity of vasoconstrictor prostanoids may be increased in human atherosclerosis. In contrast, assessment of the importance of EDHF has been hampered by the failure to conclusively identify it and therefore any role in atherosclerosis is unclear. EDHF appears to be more important as a dilator in resistance vessels that determine blood pressure, but are not themselves particularly prone to atherosclerosis.

## 1.11 Objectives of this Thesis

The objectives of this thesis are:

- To document the vascular effects of an acute change in inflammatory markers in a human *in vivo* model
- To explore the mechanism of acute human inflammation-induced endothelial dysfunction
- To specifically explore the role of oxidant stress in the development of endothelial dysfunction during inflammation
- To explore the direct vascular effects of C-reactive protein *in vitro* using highly purified protein

These areas will be covered in the subsequent chapters.

# 2 Methods

In this chapter I have described the methods used in this thesis. Detailed protocols will be described in each of the data chapters, so only outlines of the methods are included in this section.

## *2.1* Assessment of vascular function *in vitro*

Although studies in humans *in vivo* provide the most physiologically relevant model they are not always practical or appropriate. In order to study the changes caused by one isolated system it is necessary to explore the effects in *in vitro* models. Without specific inhibitors it is not always possible to divorce one event from the milieu in which it occurs to determine the relative importance of each component. This work describes the development of an *in vitro* model to study the effects of a raised concentration of C-reactive protein on blood vessels. Completing these studies in *in vitro* human vessels is very difficult due to the lack of a steady supply of suitable tissue and the heterogeneity of the material as a result of its complex previous *in vivo* exposures. As a result the majority of the work described is performed using a rat model, though the phenomenon is demonstrated with human vessels to show an absence of interspecies differences.

### 2.1.1 Vessel harvesting and incubation

#### 2.1.1.1 Rationale

Studies in animals *in vivo* do not allow the observation of the effects of one individual mediator without a specific inhibitor or genetic manipulation. Vessels removed quickly after sacrificing the animal, or during coronary bypass grafting in humans, and handled appropriately will continue to function for a reasonable period of time. During this time the vessels can be exposed to different conditions to observe the effects on their function. By dividing the vessels into a number of 2-3mm rings prior to exposure allows the comparison of untreated and actively exposed samples.

#### 2.1.1.2 Basic Protocol

Animal experiments were carried out on male Sprague-Dawley rats (200-250g) reared by Central Biological Services (University College London) on standard chow with water ab libitum. Animals were killed after stunning and cervical dislocation. The thoracic aorta was immediately exposed and removed in its entirety with minimal trauma to maintain an intact endothelium. After removal the aorta was transferred to pre-oxygenated Dulbecco's Modified Eagle Medium (with L-glutamine, 1000mg/L D-glucose and sodium pyruvate; GibcoBRL, Paisley, UK: DMEM) and extraneous fat and connective tissue removed. The vessel was then cut into 2-3mm rings and incubated in a 24 well plate for 4 hours at $37^0$C, 95%$O_2$/5%$CO_2$. Each ring was incubated in a total of 0.5ml of solution made up of fresh DMEM and the specific treatments as detailed in later sections (6.2).

Human internal mammary arteries (IMA) were obtained at the time of coronary artery bypass surgery (CABG). All subjects gave informed consent prior to surgery and the University College London Ethics Committee approved the study. Mr S Kolvekar performed all operations at the Heart Hospital (UCLH NHS trust) on patients with indications for CABG. Operations were performed according to normal clinical procedures and small sections of IMA were harvested at the end of the grafting procedure only if they were excess to the clinical need. Mr Kolvekar did not routinely use vasodilators in the preparation of his arterial conduits. Harvested tissue was immediately collected into chilled oxygenated DMEM. Vessels were then cleaned and cut into 2-3mm rings and incubated in the same way as rat aorta.

## 2.1.2 Organ bath pharmacology

### 2.1.2.1 Rationale

Measuring isometric tension of isolated vessels in an organ bath is a widely used technique to assess vascular function([447]). This technique allows the study of pharmacological effects in the absence of the influences of haemostatic mechanisms, the autonomic nervous system and physical interactions, such as changes in shear stress. Tissues are suspended in a physiological oxygenated solution to allow preservation of function and can be interrogated with known concentrations of agonists and antagonists to explore changes in isometric contraction. As it is possible to treat multiple rings from the same vessel in different baths simultaneously this technique lends itself to exploring the effects of different pre-treatments. In this work the technique was used to study the

different effects of endothelium-dependent and independent agonists on vascular rings pretreated with CRP or other inflammatory products.

### 2.1.2.2 Basic Protocol

All organ baths were cleaned prior to studies with 10% hydrochloric acid and 30% ethanol followed by multiple washes with purified water (19Ω). Organ baths were then filled with Krebs solution (composition (mM): $Na^+$ 143; $K^+$ 5.9; $Ca^{2+}$ 2.5; $Mg^{2+}$ 1.2; $Cl^-$ 128; $HCO_3^-$ 25; $HPO_4^{2-}$ 1.2; $SO_4^{2-}$ 1.2; D-glucose 11) that was maintained at $37^0$C and bubbled with 95%$O_2$/5%$CO_2$. Each channel was calibrated with a standard 5g weight prior to mounting tissues. Vessel rings were suspended, with the minimal stretching possible, between a fixed stirrup and one connected to a force transducer (FT03, Grass Transducer, USA) connected to a MacLab (AD instruments) attached to a Macintosh Computer using Chart for Macintosh. Rat aorta were pre-tensioned to 1g and IMA to 2g initially and during a 60-minute equilibration period the tension was adjusted, by means of a micro-adjuster on the fixed stirrup, to maintain this level. All rings were returned to these tensions between studies. The organ bath set up is shown in the figure below (figure 2-1).

Following the period of equilibration the maximal contraction of each vessel was determined by contraction to potassium chloride (48mM), which was repeated to ensure consistency of effect. Vessels were washed and re-equilibrated before endothelial integrity was confirmed by initially constricting with phenylephrine (PE; $10^{-7}$M) and subsequent relaxation with acetylcholine (ACh; $10^{-6}$M); relaxation by >50% indicated an intact endothelium and other vessels were

discarded. Vessels were re-equilibrated again prior to the specific protocols that are described in the relevant data chapters.

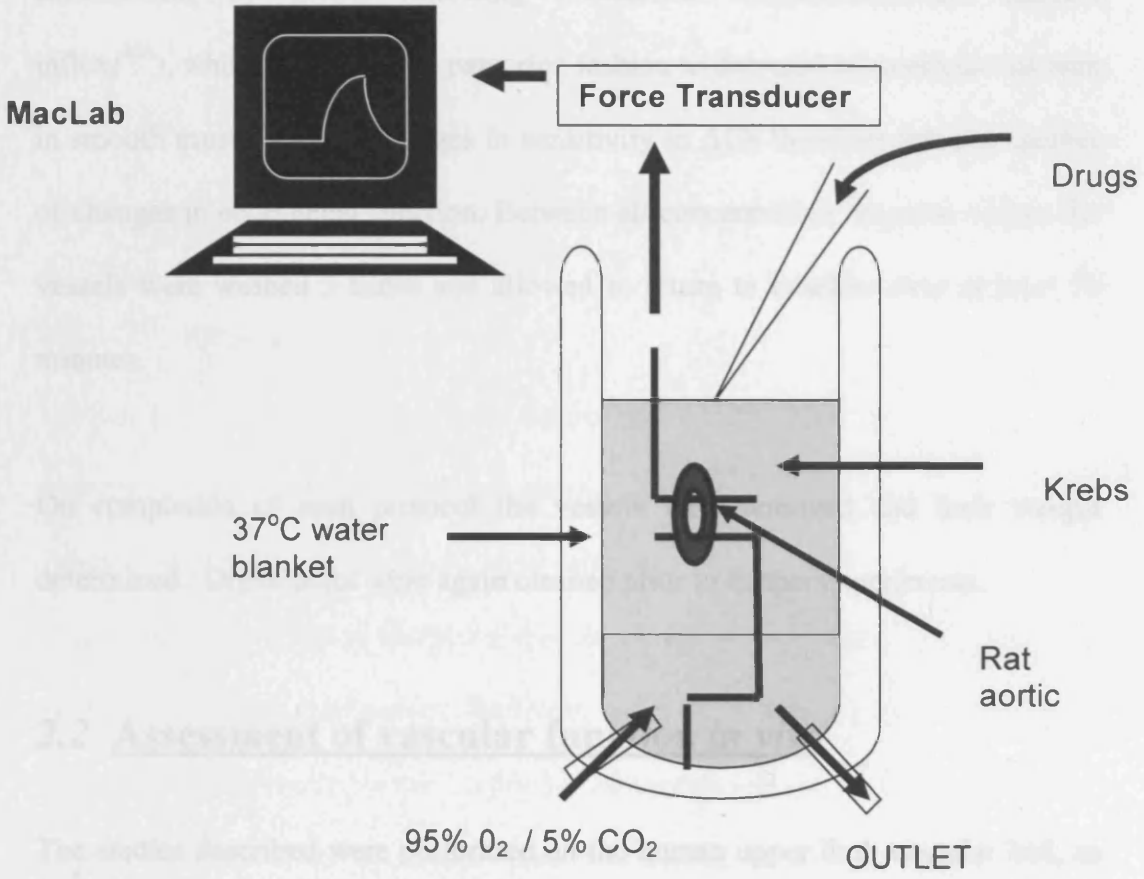



**Figure 2-1: Diagram of organ bath experimental set up**

In general a concentration response curve to PE was initially generated by cumulative addition of stock solutions of PE ($10^{-6}$-$10^{-1}$M) to achieve half log unit changes at each step. This allowed the addition of 25-50μL at each step to a total volume of 25mL – thus not significantly altering the total volume over a standard concentration-response curve. PE was selected as the standard vasoconstrictor due to its ability to selectively stimulate α adrenoceptors, and because it is easy to

wash out of the organ bath. In studies where an ACh concentration curve was generated, the vessels were initially pre-constricted with PE to 80% of the $EC_{max}$ for PE and allowed to stabilise before the cumulative aliquots of ACh were added. In these vessels, ACh largely acts by increased production of nitric oxide from the endothelium by eNOS following muscarinic receptor-mediated calcium influx([448]), which then acts in a paracrine fashion to decrease intracellular calcium in smooth muscle cells. Changes in sensitivity to ACh therefore act as a marker of changes in endothelial function. Between all concentration response curves the vessels were washed 3 times and allowed to return to baseline over at least 30 minutes.

On completion of each protocol the vessels were removed and their weight determined. Organ baths were again cleaned prior to further experiments.

## *2.2* Assessment of vascular function *in vivo*

The studies described were performed on the human upper limb vascular bed, as this is readily accessible and a suitable surrogate for disease-relevant vessels. Selective cannulation of the brachial artery at the antecubital fossa allows the observation of changes in the resistance vascular bed following the infusion of pharmacological agents. Specific agents can be used to explore changes in blood flow that are dependent on signalling from the endothelium and those that are dependent on smooth muscle integrity. The same route can be used to assess the effects of high local concentrations of components of the nitric oxide pathway. In other studies high-resolution ultrasound of the brachial artery was used to detect changes in the vessel diameter, a response that is dependent on NO

bioavailability. These models have been used in a large number of studies by a variety of groups worldwide. Venous occlusion plethysmography was the preferred method only when local infusions of agonists were required, as otherwise flow mediated dilatation allowed for repeated measures without any inherent risk of arterial cannulation.

## 2.2.1 Venous occlusion plethysmography

### 2.2.1.1 Rationale

Venous occlusion plethysmography allows the investigation of the tone of the resistance vasculature within the forearm. This technique measures the rate of increase in forearm volume occurring when the venous outflow is temporarily obstructed. Arterial blood continues to enter the forearm and consequently the arm expands as blood accumulates. Forearm volume is derived from changes in forearm circumference by mercury-in-silastic strain gauges. The rate of blood flow is represented by the initial linear rate of rise in forearm volume, and is quantitated from the gradient of the slope transduced from the strain gauges.

Pharmacological agents can be infused locally at doses 20-100-fold lower than would be required to produce a systemic effect. As blood vessel responses within the hand are largely dependent on skin blood flow, excluding the hands with wrist cuffs inflated to suprasystolic pressures reduces variability during the recording period. Simultaneous recording of blood flow in the contra-lateral (uncannulated) arm allows for correction for systemic changes, such as alteration in sympathetic tone, during recording. As a consequence data from cumulative dose response

curves is presented as percentage changes in the ratio of blood flow measured simultaneously in both arms([449]).

### 2.2.1.2 Basic protocol

All studies were performed on healthy volunteers who were taking no medications, did not have a family history of premature coronary artery disease and were non-smokers. Subjects were supine in a temperature-controlled laboratory with both forearms raised to above the level of the heart to allow efficient venous drainage. Drugs were administered in saline (sodium chloride 0.9% (w/v)) and infused using an infusion pump (Harvard) at 0.5 ml/min through a 27G needle inserted into the non-dominant brachial artery (Cooper's Needle Works, UK) under local anaesthetic (lignocaine 1%). Blood flow was allowed to return to baseline for 30 minutes following cannulation before recordings were made and for 15 minutes between each dose response curve. During recordings the hands were excluded from the circulation by wrist cuffs inflated to 200mmHg([450]). Upper arm congesting cuffs were inflated to 40mmHg for 10 seconds of every 15-second recording cycle. Measurement of forearm circumference was simultaneously made in both arms by mercury-in-rubber strain gauges connected to a plethysmograph and recorded electronically (Maclab, UK). The strain gauges were calibrated at the start of each experiment. The initial linear slope of the last four recordings of both arms during baseline and each subsequent infusion were collected. These were exported to a spreadsheet (Excel) and the ratios of infused to non-infused arms were calculated and then averaged over the four recordings. The percentage change in blood flow due to increasing doses of

drugs over the baseline was calculated and plotted in a separate graphics program (GraphPad Prism).

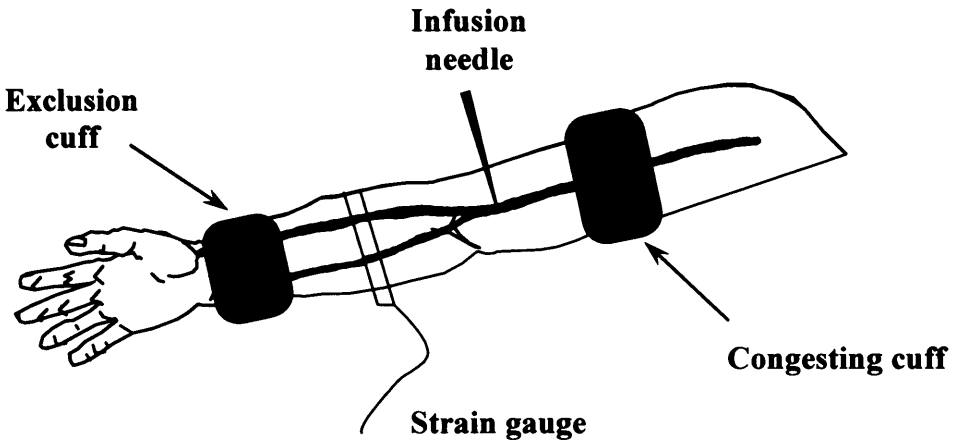



**Figure 2-2: Diagram of set up of active forearm for venous plethysmography (control arm lacks infusion needle)**

## 2.2.2 Flow mediated dilatation

### 2.2.2.1 Rationale

Although it is widely used and very reproducible venous occlusion plethysmography has the disadvantage of requiring an intra-arterial needle to probe vascular reactivity pharmacologically. An alternative technique is to measure the change in arterial diameter induced by a change in blood flow([107;451]). Endothelial cells release nitric oxide (NO) and other endothelium-derived relaxing factors in response to mechanical shear stress. The precise mechanisms for the acute detection of shear forces and subsequent signal transduction to modulate

vasomotor tone are not fully understood, but probably involve calcium-activated potassium-channel opening, membrane hyperpolarization and calcium-mediated activation of endothelial nitric oxide synthase. *In vivo* the endothelium can respond to shear stress due to increased flow, by releasing NO that causes relaxation of the underlying smooth muscle and vasodilatation([452]). In humans, in vivo, it is possible to image the radial, brachial, femoral or tibial arteries with high-resolution ultrasound in longitudinal section. Direct visualisation of the brachial artery with ultrasound allows the detection of changes in the vessel's diameter in response to this shear stress induced endothelial-dependent nitric oxide release. Unlike plethysmography that measures changes in resistance vessel tone, this technique directly visualises changes in conduit arteries. As a result caution is needed in comparing results between techniques.

Although originally the measurement of the arterial diameter was performed using callipers with B-mode recordings this has evolved to the use of quantitative ultrasound techniques and customised edge-detection software. This allows the serial recording of multiple time points during a study and the production of a dilatation curve for each individual from which the change in diameter can be determined.

An increase in shear stress is achieved by the production of reactive hyperaemia following a period of ischaemia in a distal vascular bed. To control for non-specific smooth muscle effects changes in the diameter of the reference artery can be studied following systemic nitrate vasodilators. Each study is performed after

the vessel had been allowed to return to the same baseline diameter as variation in the baseline diameter has been shown to affect the degree of dilatation([107]).

#### 2.2.2.2 Basic protocol

All studies were performed on healthy male volunteers who were taking no medications, did not have a family history of premature coronary artery disease and were non-smokers. Subjects were supine in a temperature-controlled laboratory and in all cases the right arm was studied. Three ECG leads were attached to the chest for continuous ECG recording. After 10 minutes rest and recording a baseline blood pressure, oral and peripheral temperature and pulse rate the arm was abducted horizontally and placed in a cradle and a blood pressure cuff positioned around the mid-forearm. The brachial artery was imaged longitudinally in the upper arm (5-10cm above the cuff) with high-resolution vascular ultrasound (Acuson XP10, 5-10 MHz broad bandwidth linear array transducer) with adjustments in the depth and gain settings made to ensure adequate visualisation of the anterior and posterior vessel wall over as long a section as possible. The ultrasound probe was then fixed in place with a stereotactic clamp that allowed small adjustments to maintain the image during the procedure. The position of the probe was marked after each study and images printed to ensure that at future sittings the same portion of the vessel, with the same baseline diameter, could be visualised. Ms Clare Storry, a technician at the Institute of Child Health, London, performed the ultrasound studies for this work.

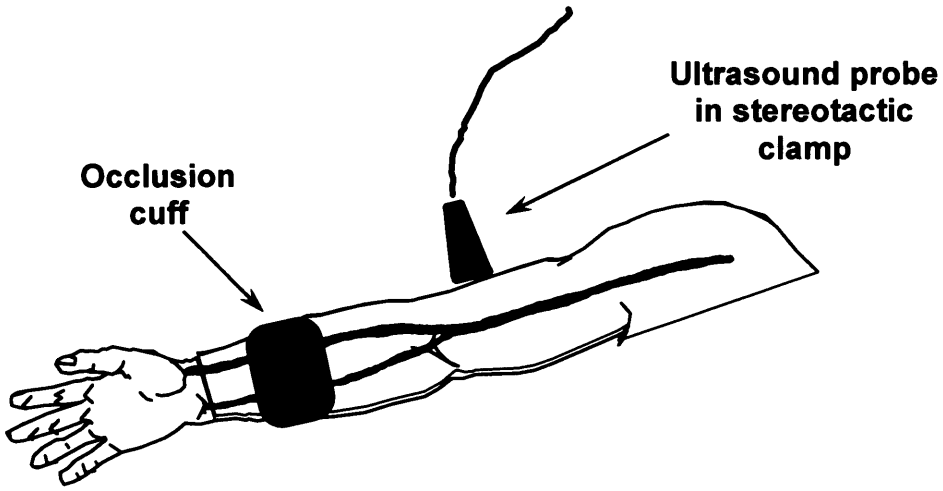



**Figure 2-3: Diagram of set up for flow mediated dilatation study.**

Pulse wave Doppler was used to measure blood flow velocity expressed as the velocity time integral (VTI; average distance moved by a red cell in one cardiac beat) with the cursor set at 70 degrees to the longitudinal axis of the artery with the range gate set to 1.5mm. Although there are inherent limitations in this assessment relative changes are accurate. The B-mode image was set to update on the R wave of the ECG and longitudinal end-diastolic images were acquired to a PC every three seconds over a 2-3cm segment of the vessel.

Following an initial 60-second base line recording the forearm cuff was inflated to suprasystolic pressure (300mmHg) for 5 minutes to occlude flow to the hand and provide a hyperaemic stimulus on deflation. Following deflation recordings were

continued for 5 minutes. Any change in blood pressure was recorded and then a two minute rest occurred before a further 60-second baseline recording. Sublingual GTN (25mcg) was administered sublingually and changes in vessel diameter were recorded for a further 5 minutes before a final recording of the blood pressure was made.

All scans were analysed off-line, using customised software with an automated edge detection algorithm (Brachial Tools, Iowa, USA), to determine the maximal change in diameter in response to both changes in flow, endothelial-dependent, and secondary to sublingual GTN, endothelial-independent. These are expressed as a percentage change from the minute long baseline. Following each study the baseline diameter, haemodynamic data, maximal flow mediated dilatation, maximal GTN-induced dilatation and the change in velocity-time integral was recorded.

## 2.3 Human model of systemic inflammation

In order to study the effects of an acute inflammatory episode, a model using typhoid vaccination has been developed([324]). This allows the study of different subjects and the use of different protocols in a safe and reproducible fashion.

### 2.3.1 Inflammatory stimulus

Inflammation was induced by the injection of Typhoid vaccine (Typhim Vi) as this produces a low-grade inflammatory response, as evidenced by a rise and fall in cytokine levels without a systemic haemodynamic response that could interfere with the interpretation of changes in blood flow.

In all cases studies were repeated in the same individuals 16 hours before and 8 hours after the vaccination with Salmonella typhi capsular polysaccharide vaccine 0.025mg (Typhim Vi, Pasteur Merieux MSD) given into the gluteus maximus muscle. In some cases further studies were repeated at 32 and 56 hours after the stimulus.

## 2.4 Cell culture models

### 2.4.1 Primary cell lines

#### 2.4.1.1 Rationale

As differences exist in the basal levels of CRP in different species and in their properties and quaternary structure, as described earlier (section 1.8.2), it is important to attempt to perform experiments in the same species as the CRP is sourced. As experiments in this thesis had been done with human internal mammary arteries, but the supply of tissue was difficult, it was important to also investigate effects on protein expression in a human cell line. Human coronary artery endothelial cells were chosen due to the appropriateness of their lineage to atherosclerotic disease, the avoidance of cross-species effects and their ready availability within the laboratory. Cells were exposed to a low inflammatory level of CRP (limited by the concentration of the samples used to 50mg/L) and after 24 hours harvested and protein expression studied.

### 2.4.1.2 Protocol

Passage five human coronary artery endothelial cells (HCAECs; Promocell, Heidelberg, Germany) were grown to 80% confluence in six well plates with supplier's growth media (supplemented with fetal calf serum (2%), amphotericin B (0.05mg/ml) and gentamicin (50μg/ml)) at 37°C in 95%$O_2$/5%$CO_2$. Achieving 80% confluence took on average 4 days and the media was replaced on alternate days. All procedures were performed under a sterile positive-pressure hood and cells were checked for integrity by light microscopy before and after each step.

Cells of earlier passage were grown to confluence in $T^{25}$ flasks and then washed with phosphate buffered saline (PBS) and mobilised with 1ml of trypsin solution. Following addition of the trypsin solution the plates were incubated for 1 minute and gently agitated to release the cells (confirmed by light microscopy). Before being aliquoted into fresh six well plates (2.5ml per well) or a fresh $T^{25}$ flask (3.5ml) the trypsin was neutralised with the addition of an excess of culture media (2.5ml if for return to $T^{25}$ flask and 17ml if for addition to six well plates). In this way for each experiment all cells were grown for the same duration and were of the same passage.

Media was then removed and stored and cells were washed with PBS before incubation for 24 hours with fresh media containing pure human CRP (final concentration 50mg/l), commercial human recombinant CRP (Calbiochem; 50mg/l, containing sodium azide, final concentration 380μM), or an equal volume of buffer (TRIS buffer without sodium azide) to a total volume of 1ml per well. All treatments were performed in triplicate and all experiments were also done in triplicate.

Following incubation cell integrity was confirmed by light microscopy, and by survival assay in some cases (described below; 2.4.1.3), and conditioned media was then removed and stored at -80°C until analysis. Cells were lysed with 200µl RIPA buffer (1%w/v Nonidet, 1%w/v sodium deoxycholate, 0.1% w/v SDS, 0.01M sodium phosphate with EDTA-free complete protease inhibitor; Roche), disrupted by sonication and collected by centrifugation (10 minutes at 14,000g) for assays of protein expression as described in section 2.5.1. Equivalence between groups was achieved by cell counting and protein quantification assay of lysed cells.

### 2.4.1.3 Cell viability assay

In order to confirm that treatment with different agents does not cause a change in cell viability a cell proliferation assay was performed. This was performed with Thiazolyl Blue Tetrazolium Blue (MTT), which is a yellow solution, which is converted to water-insoluble MTT-formazan, a dark blue substance, by mitochondrial dehydrogenases in living cells([453]). The blue crystals are then solubilized and measured colorimetrically. The optical density of the resultant samples reflects the quantity of viable cells and therefore confirms whether culture treatments alter cell survival.

Following incubation of cells for 24 hours with control and active media this was replaced, under sterile conditions, with 2ml of fresh media containing MTT (0.2mg/ml; Sigma) and the cells were incubated for a further 60 minutes at 37°C, 95% $O_2$/5% $CO_2$. The media was discarded and replaced with 400µl of dimethyl

sulfoxide (Sigma), before a further 5 minutes incubation. Finally 100µl of solution was transferred, in triplicate, to a 96 well flat-bottomed plate and the optical density read at 490nm on an automated plate reader. Results were averaged over the triplicates and expressed as optical density for each treatment.

## 2.4.2 Transfected cell lines

### 2.4.2.1 Rationale

In an attempt to explore the effects of CRP on the pterin pathway, modified murine endothelioma cell (sEnd-1) lines were employed. This was a model that had been used by Dr M Nandi, another member of the group, to look at the changes in pterins following stimulation with lipopolysaccharide and inflammatory cytokines. In this model two strains of sEnd-1 cells were used, the first had been transfected with an empty pcDNA vector and the second with a gene to over express GTP cyclohydrolase-1 feedback regulatory protein (GFRP), the endogenous inhibitor of GTPCH-1. In the stimulated cells an increase in nitrite and nitrate, by the Griess reaction, had been observed that was markedly attenuated in the cells over-expressing GFRP. To explore the effect of CRP on this system, particularly to look at the differences between commercially available preparations and the in house extraction, similar experiments were performed. As changes were seen in the colour of the media of cells treated with the different protein preparations (which is pH dependent due to its phenol red content), the pH of the media were quantified by a colourimetric technique.

### 2.4.2.2 Protocol

The preparation and transfection of the cells described in the next two sections were performed by M Nandi, a member of our department, as part of her PhD.

#### *2.4.2.2.1 Expression and cloning of human kidney GFRP*

First strand cDNA synthesis of GFRP was performed using human kidney poly $A^+$ RNA (Clontech) as a template and was primed using an oligonucleotide complementary to the 3' UTR of human GFRP [GGT GCC CCG TCT GCA AATC]. PCR amplification of the cDNA was performed using oligonucleotides complementary to nucleotides 3-17 and 255-236 of the GFRP mRNA (94°C, 10secs; 55°C, 10secs; 72°C, 30 secs; 30xcycles) yielding a 0.25 kB product which was subsequently gel purified (Qiagen), TA cloned into pCR TOPO 2.1 (Invitrogen) and sequenced to confirm that no mutations had been introduced during amplification.

In order to construct an N-terminally Myc tagged form of GFRP into the mammalian expression vector pcDNA 3.1 Hygro (Invitrogen) PCR amplification of the TA cloned cDNA was performed using a 5'oligonucleotie primer encoding a HindIII restriction site (in italics) followed by the 9E10 N-Myc epitope (in bold) [GATC *AAG CTT* **ACC ATG GCC GAA CAA AAA CTC ATC TCA GAA GAG GAT CTG GGC GGC** CCC TAC CTG CTC ATC AGC ACC]. The 3' oligonucleotide primer contained a downstream Xho 1 site (in italics) [CTGA *CTC GAG* TCA CTC CTT GTG CAG ACA CCA C]. PCR amplification (94°C, 10secs; 40°C, 10secs; 72°C, 30 secs; 20xcycles) produced a band approximately 0.35kB in size. This product was gel purified, ligated into pcDNA 3.1 Hygro (pre

digested with Xho I and Hind III), and transformed into E. Coli sub cloning efficiency cells (DH5α – Invitrogen). Recombinant plasmids were selected and the insert verified by sequencing.

#### *2.4.2.2.2 Tranfection of sEnd 1 cells*

Murine endothelioma cells (sEnd 1)([454]) were grown to confluence in a 6 well plate in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) containing 10% heat inactivated fetal bovine serum, 100 units penicillin G sodium and 100ug streptomycin sulphate (Invitrogen) and 2mM L-glutamine (Invitrogen) in a humidified incubator (37°C/ 5% $CO_2$).

Either GFRP/pcDNA 3.1 Hygro or the pcDNA 3.1 Hygro (empty vector control) was transfected into sEnd 1 cells using TFX 20 Transfast transfection reagent (according to manufacturers instructions). 48 hours post transfection, medium was replaced with medium containing 500mg/ml hygromycin (Roche) to select for transfected cells. Following five days of culture in the selective medium, individual hygromycin colonies were isolated and transferred to 96 wells plates for expansion. To confirm expression of N-terminally Myc tagged GFRP, lysates of cells were resuspended in RIPA buffer and resolved by SDS PAGE and analysed by western blotting using the 9E10 monoclonal α myc antibody (1:1000).

#### *2.4.2.2.3 Incubation with C-reactive protein*

Cells were grown on 24 well plates in DMEM in the same way as described for HCAECs, with antibiotics to maintain pure cell lines. Cells of passage 5-7 were mobilised from $T^{25}$ flasks with 1ml of trypsin solution and then after neutralisation of the trypsin with the addition of 23ml of DMEM aliquoted in equal volumes into a fresh 24-well plate. These were grown to 80% confluence in an incubator (37°C in 95%$O_2$/5%$CO_2$) with media being changed on alternate days. For each experiment one plate of GFRP overexpressing cells (B1) and one of empty vector cells (pcDNA) were used and treated in the same way. Generally the pcDNA cells grew more quickly than the B1 cells and this was accounted for by plating out the cells more sparsely before the incubation period and by correcting all NO assays for the protein content of each well as described below (section 2.5.1.2.2). As with the HCAECs all procedures were carried out with sterile equipment in a sterile positive pressure hood.

At the start of the experiments 100µl of solution from each well was aliquoted into a 96 well plate and this was frozen (-80$^0$C) for 24 hours in order to analyse all the samples at the same time. The cells were washed with PBS and active media added. Two incubation protocols were performed, with each treatment being applied to 4 wells from each plate and performed in triplicate.

The first set of experiments consisted of purified CRP (50mg/L), commercially available CRP (Calbiochem; 50mg/L), purified CRP which had then been depleted by incubating with sepharose beads coated with phosphoethanolamine (final concentration <0.01mg/L), control buffer solution, a cytokine mixture as a

positive control (10ng/ml TNFα, 100unit/ml interferon γ and 5μg/ml LPS) and a negative control with no additions.

In the second set of experiments the buffer solution group was replaced by commercially available CRP, which had been dialysed against a large volume of sodium azide-free buffer to remove this contaminant (final CRP concentration 50mg/L). On two occasions a further plate consisting of 12 wells with pcDNA and 12 with B1 cells was run containing media alone or one of two concentrations of the metabolic poison myxthiazole (10μM or 1μM).

Plates were incubated for 24 hours at 37°C in 95%$O_2$/5%$CO_2$ and then 100ul of media, in duplicate, was drawn off into the 96 well plate from the first day for the performance of a Greiss assay as detailed in the biochemical methods below (section 2.6.5). A further 100□l was drawn off into a second 96 well plate for the determination of pH, also detailed below (section 2.6.6), and a final 200□l was saved into eppindorfs for use on pH indicator strips. The remainder of the media was discarded and the cells were lysed with RIPA buffer (as described previously) and the total protein content of each well was determined, as indicated in section on western blotting (2.5.1.2.2), in order to allow correction of the NO production assays for the amount of cellular material in each well.

## 2.5 Molecular biological techniques

### 2.5.1 Western blotting

#### 2.5.1.1 Rationale

Western blotting (immunoblotting) allows the identification and semi-quantification of proteins within biological samples based on their electrophoretic properties. Equal quantities of protein are added under denaturing conditions to a polyacrylamide gel matrix and are separated according to their molecular weight as they move by one-dimensional electrophoresis towards the anode. By the use of a coloured protein ladder of known molecular weights it is possible to identify individual products. Specificity is improved by transferring the samples after separation to a nitrocellulose transfer membrane that fixes their relative position. This can then be interrogated with antibodies specific to the protein of interest. For practical reasons the antibody process is generally performed in two stages; firstly a specific antibody is used and then secondly an antibody to this, tagged with horseradish peroxidase, is applied. The presence of horseradish peroxidase allows the detection of the antibody by its emission of light under specific conditions, which is captured on radiographic film. Although only semi-quantitative the relative intensity of bands demonstrated by this technique indicate the degree of expression of the protein studied provided that equal quantities of total protein have been added to the system and complete and equal transfer has occurred for all samples.

### 2.5.1.2 Protocol

#### *2.5.1.2.1 Sample preparation*

Prior to separation with western blotting tissue samples were processed to ensure cellular disruption without degradation of the relevant proteins. For whole vessel preparations rat aorta were incubated in the same way as detailed for organ bath experiments; that is in DMEM for 4 hours with the relevant treatment, before being snap frozen by immersion in liquid nitrogen. Tissues were then smashed in a metal pestle and mortar, which itself had been cooled to –80°C, before being re-suspended in a solution of protease inhibitors (complete EDTA-free protease inhibitor cocktail tablet, Roche, USA). Once re-suspended the samples were then exposed to repeated thaw-sonication-freeze cycles to ensure adequate disruption. Finally samples were centrifuged (10 minutes at 4000rpm) and the supernatant drawn off for further analysis.

Samples obtained from cell culture experiments were prepared in a different way to reflect the relative ease of disruption of this type of material. Incubation protocols are detailed in section 2.4.1.2. After all culture medium was removed, 0.2ml of RIPA lysis buffer (1% w/v Nonidet P-40, 1% w/v sodium deoxycholate, 0.1% SDS, 0.15M NaCl, 0.01M sodium phosphate (pH 7.2), 2mM EDTA, 50mM sodium fluoride, 0.2mM sodium vanadate, 100U/ml aprotinin) was added to each well and the plates agitated to release the cells. Disruption of the cells was aided by repeated drawing up and down through a 200μL pipette, before freezing the samples. As with vascular tissue the samples were centrifuged and the supernatant stored for further analysis.

Although western blotting is only semi-quantitative it is important to load equal quantities of protein to each channel. To achieve this sample protein contents were determined by the Bradford method. This involves quantification of the binding of Coomasssie brilliant blue to the unknown protein in comparison to different known concentrations of bovine serum albumin([455]). A protein ladder was generated with bovine serum albumin (Promega) over a range of 1-25μg/ml and 80μl added in duplicate to a 96 well plate. Unknown proteins were diluted 100-200 fold in water and 80μl added in duplicate to the plate. To each well 20μl of coomassie solution (Bio-Rad Laboratories, USA) was added and mixed. The colour change was quantified with a colorimeter set at 595nm absorbance and results compared to the standards with an appropriate correction for prior dilution. The quantity of solution added was then adjusted according to this concentration and the individual protein under investigation. Prior to loading into the stacking gel the desired quantity of sample was added to a loading buffer in a ratio of 5:1. The loading buffer (80mM Tris (w/v; BDH), 5% β-mercaptoethanol (v/v; BDH), 0.02% bromophenol blue (w/v), 10% glycerol (v/v), 2% SDS (w/v) with pH to 6.8 with HCl) served the joint purpose of allowing easy visualisation of the samples and aided in protein denaturisation and the separation of disulphide bonds. Immediately prior to loading of the samples they were boiled at 99°C for 10 minutes and then chilled on ice to ensure complete protein separation.

#### *2.5.1.2.2 Protein separation*

Separation of proteins was achieved by one-dimensional electrophoresis on denatured samples. SDS separation gels were made for each experiment, with the percentage content of acrylamide varied according to the protein of interest.

Initially two glass plates were fitted into a customised clamp separated by 0.75mm spacers and the bottom sealed with agarose. The components of the separating gel were mixed (acrylamide, 3M Tris (pH 8.8), 10% w/v SDS, water) and then TEMED (NNNN-tetramethylethylenediamine) and 10% w/v ammonium persulphate were added to set the material just prior to pouring between the plates, to approximately 2cm from the top. Diluted Tris (pH 8.8) was poured over the gel to prevent drying during the setting process.

After the separation gels had set, a stacking gel (4% acrylamide, with pH 6.8 3M Tris) was poured and a 0.75mm thick comb was inserted to produce ten channels. After this had also set the comb was removed and the wells cleaned of excess material. Two gels were produced at a time and they were then mounted as a pair within the clamping frame and electrode assembly and placed in the buffer chamber (Bio-Rad Laboratories, USA). The entire assembly was then immersed in running buffer (0.125M Tris base, 0.96M glycine, 0.5% w/v SDS, water, pH 8.3).

One or more pre-stained protein ladders (Invitrogen, USA) and the samples for separation were added to the wells within the stacking gel. No more than 30µl of solution was added to any well to avoid overfilling and spillage into neighbouring channels. Gels were then run at a constant voltage of 200V in order to separate the proteins, the duration according to the molecular weight of interest.

Following separation the gels were removed from the tanks and sandwiched between six sheets of Whatman paper and a nitrocellulose membrane (Hybond-P,

Amersham, UK) to allow transfer of the samples. Gel orientation was designated by the removal of the top left hand corner. Whatman paper was soaked in transfer buffer (3.6g Tris base, 14.4g glycine, 200ml methanol made up with water to 1000ml, pH 8.3) and nitrocellulose membrane activated with methanol prior to transfer. All air bubbles were removed and then a 200mA constant current passed across the sandwich for 30 minutes to achieve transfer. At all times the nitrocellulose membranes were handled as little as possible and with the use of gloves to avoid contamination.

Following transfer the nitrocellulose membranes were cut to size and transferred to clean baths and immersed in phosphate buffered saline with Tween (PBST; 1L of PBS made from tablets (Sigma) in sterile water and 1ml Tween 20 (Sigma)) containing milk powder (5%w/v, Marvel) and gently agitated. This milk solution acted to bind and block non-specific proteins and improve resolution of antibody identification.

Gels were stained for protein content with Coomassie, by initially immersing in staining solution (1 part acetic acid, 3 parts isopropanol, 6 parts water and 0.5% w/v Coomassie brilliant blue) for 1 hour before changing this to destaining solution (7% v/v acetic acid, 5% v/v methanol, 88% water) for 12 hours (changed 3 times), before finally fixing with fixing solution (50% v/v methanol, 10% v/v acetic acid, 40% water) and drying.

#### *2.5.1.2.3 Protein identification*

Protein identification was performed by a two-stage antibody technique involving the use of a specific primary antibody and then an enzyme-linked secondary antibody directed against the primary. The primary antibodies for eNOS and iNOS were obtained from a commercial source (rabbit anti-human eNOS (c-20) and rabbit anti-mouse iNOS (m-19)[Santa Cruz Biotechnology, California USA]), whereas the anti-GTPCH-1 anti-peptide antibody was developed for the department by Sigma using amino acids 17-45 from the human sequence in a rabbit and was polyclonal([456]) .

Following at least 4 hours of blocking in milk solution this was washed off and changed to primary antibody solution (1:1000 antibody in PBST). This was then incubated overnight on a rotary agitator at 4°C. In the case of anti-GTPCH-1 the solution contained 5% milk to reduce non-specific binding. On completion of the overnight exposure excess primary antibody was removed by washing with agitation for 10 minutes in fresh PBST. This was repeated a total of six times. Secondary antibody exposure was performed for 2 hours at room temperature, before a final six further wash cycles. The secondary antibody was anti-rabbit IgG fused with horseradish peroxidase (Santa Cruz Biotechnology, California USA) diluted in PBST to a final concentration of 1:3000.

Development of the bound secondary antibody was achieved with the ECL plus system (Amesham, UK) that is based on the enzymatic production of an acridinium ester, by horseradish peroxidase, that emits light. The light produced was recorded onto photographic film exposed for increasing durations from 5

seconds to 2 minutes. The exact durations were optimised for the individual assays. Localisation of individual bands was achieved by comparison to a known standard and relative to the pre-stained protein ladder.

Developed films were scanned onto a computer with a flat-bed scanner once appropriately labelled. Although a semi-quantitative technique, the intensity of the bands were compared with the aid of Scion imaging software (Scioncorp, USA) and averaged over three individual blots.

Membranes were Coomassie stained in the same way as gels, without the fixing step, and dried and also scanned onto a computer to allow quantification of the degree of protein transfer in each channel.

## 2.6 Biochemical techniques

### 2.6.1 Isolation of human C-reactive protein

#### 2.6.1.1 Rationale

Although preparations of CRP are available from commercial sources they are primarily intended as standards to calibrate assays for serum CRP. As a consequence they include sodium azide to retard the growth of any contaminating bacteria, as it acts as a mitochondrial respiratory chain inhibitor([276;278]). The presence of this metabolic poison therefore makes commercially available CRP unsuitable for functional experiments in cells or intact tissues. In addition, the purity of commercially available CRP has been questioned, particularly with regard to the presence of immunoglobulins([249]). As a result, for these studies,

collaboration was made with Professor M B Pepys' group at the Centre for Amyloidosis and Acute Phase proteins at the Royal Free Hospital (specifically Dr G M Hirschfield) to obtain CRP purified from human ascitic fluid, which specifically did not contain sodium azide as part of its buffer. In addition all experiments were then performed with the buffer (0.01M Tris 0.138M NaCl 0.002M $CaCl_2$, pH 8) used for the CRP as the negative control.

### 2.6.1.2 Protocol

Human CRP was purified from human ascites by affinity chromatography as previously described([457]). Briefly, the ascites was filtered and then Sepharose-phosphoethanolamine beads were used to capture the human CRP, which was subsequently packed into a column and the CRP eluted off with TrisNaCl-Ca buffer containing 1mM phosphocholine (pH 8). The CRP was then dialysed extensively into TrisNaCl-Ca buffer, containing no azide (pH8), to remove the phosphocholine (final concentration 2mg/ml). C-reactive protein was demonstrated to be in its native pentameric form by size exclusion chromatography, and functionality was confirmed by calcium dependent binding to immobilised phosphoethanolamine. SDS gel electrophoresis demonstrated a single band corresponding to an approximate size of 23K. CRP and its vehicle contained <0.5ng/ml bacterial lipopolysaccharide (LPS) by Limulus chromogenic assay (BioWhittaker Europe).

## 2.6.2 Urine and blood sampling

### 2.6.2.1 Rationale

Our group have previously shown that the inflammatory response to vaccination leads to endothelial dysfunction([50;324]). Collection of samples following vaccination allows the investigation of the size and type of an inflammatory response, an exploration of changes in cardiovascular risk factors and an assessment of the temporal change in inflammatory markers and indices of endothelial function.

### 2.6.2.2 Protocol

During plethysmography studies, described in chapters 4 and 5, blood and urine samples were taken before and 2 hourly for 8 hours after vaccination for the measurement of serum albumin and plasma lipids and urinary protein. As a control, urine samples were taken at 4pm on a different day in the absence of vaccination. In the studies using ascorbic acid prior to vaccination (chapter 4) samples of blood only were collected on day 1, prior to vaccination and 4 and 8 hours after. For FMD studies (chapter 3) blood samples were taken prior to each study and additionally just before vaccination and again 4 hours later.

Blood was collected into tubes containing lithium heparin, citrate and EDTA and plasma was obtained by centrifugation (3000g for 10 minutes), then aliquoted and stored at $-20^{0}$C. Plasma was used to measure IL-6 and IL-1Ra with a commercially available ELISA as detailed below (section 2.6.3) (R and D systems, Abingdon, Oxon). Plasma was analysed at baseline and 8 hours

following vaccination for concentrations of total cholesterol, HDL and albumin by reflectance spectroscopy (Vitros multichannel analyser 250,700,750) by a hospital laboratory (Great Ormond Street) and for total anti-oxidant capacity as detailed below (section 2.6.4). Urine was collected in sterile containers and chilled and frozen at –20°C immediately. Samples at baseline and 8 hours after vaccination were analysed for urinary albumin by immunonephlometry (Behring BNII analyser) and creatinine concentrations by enzymic assay and chemiluminescence (Vitros 700, Ortho Clinical Diagnostics) by a hospital laboratory (Great Ormond Street).

In the study with pre-treatment by ascorbic acid samples were collected into plain tubes, EDTA and lithium heparin. Plain tubes were allowed to clot and then all samples were centrifuged (at 3000g for 10 minutes). A further EDTA treated sample of whole blood was frozen (-20°C) for analysis of red blood cell glutathione. Serum (500μL) was added to equal volume of fresh 10% metaphosphoric acid and recentrifuged and the supernatant frozen. Plasma with EDTA (1ml) was deproteinated with 30% perchloric acid (60μL) and the supernatant stored. The remainder of the samples were stored at –80°C for analysis of markers of oxidant stress as detailed below (section 2.6.4).

Analysis by ELISA was made of IL-6, IL-1Ra and neopterin and CRP by automated immunoassay as detailed below (section 2.6.3).

### 2.6.3 Blood analysis by immunoassay

#### 2.6.3.1 Rationale

All the assays used employed the quantitative sandwich immunoassay technique whereby a relevant monoclonal antibody was pre-coated on to a multi-well plate. Samples, and standards, bind to this immobilised antibody and after removal of unbound substances a specific enzyme-linked antibody was added that binds to the immobilised protein. Following a further wash a substrate solution was added and the colour change quantitated to determine concentration.

IL-6, IL-1Ra and TNFα were assayed, as they are inflammatory cytokines involved in the immune response to vaccination([324;458;459]). They represent an early and sensitive marker of inflammation and IL-6 in particular is involved in the development of the acute phase response. CRP was measured as it is the classical acute phase protein and as described in the introduction is predictive of future cardiovascular complications (section 1.8.1). Neopterin, itself an acute phase protein, was assayed to reflect changes in the pterin pathway as it is also generated following upregulation of GTP cyclohydrolase-1 which is the rate limiting step for these products.

I performed the assays of the interleukins and neopterin, with the exception of the 5 subjects in the first venous occlusion plethysmography protocol. A member of Dr Vidya Mohamed-Ali's group performed the remainder of the IL-6/IL-1Ra analysis and Ruth Gallimore in Professor MB Pepys' laboratory performed the CRP assays.

### 2.6.3.2 Protocols

#### *2.6.3.2.1 Interleukin-6*

Assays were performed using a pre-packaged kit (R and D Systems, Abingdon, Oxon) with all samples being studied in duplicate in a 96-well plate according to the manufacturer's instructions. Assay diluent (100μL) and standards (over a range of 0.156 to 10pg/mL) or citrated plasma samples (100μL) were added to the plates coated with mouse monoclonal antibody against IL-6 and incubated on an orbital shaker for 2 hours. The plates were then washed six times with a non-phosphate containing buffer before IL-6 conjugated to alkaline phosphatase was added (200μL), before incubation and agitation for a further 2 hours. A further six washes were performed before substrate solution (NADPH with stabilisers; 50μL) was added to each well and the plate incubated for 60 minutes on the desktop. Amplification was performed for 30 minutes after addition of the relevant amplifier solution (alcohol dehydrogenase and diaphorase; 50μL). NADH, formed from NADPH by reaction with the fixed alkaline phosphatase in a dose dependent fashion, reduced a tetrazolium salt to produce a coloured formazan dye (and NAD+ then is recycled in the amplification step). The reaction was stopped by the addition of stop solution (2N sulfuric acid; 50μL) and the plates immediately read in a microplate reader at 490nm (with wavelength correction at 690nm). Samples were compared to the standard curve and a final concentration obtained, which was averaged over the duplicates. The manufacturer's lower limit of detection of this assay was 0.039pg/mL, with an interassay coefficient of variation (CV) of 7.2% at 2.78pg/mL and 6.5% at 5.65pg/mL.

#### *2.6.3.2.2 Interleukin-1Ra*

Assays were performed using a pre-packaged kit (R and D Systems, Abingdon, Oxon) with all samples being studied in duplicate in a 96-well plate according to the manufacturer's instructions. Assay diluent (50μL) and standards (over a range of 46.9 to 3000pg/mL) or lithium heparin plasma samples (200μL) were added to the plates coated with mouse monoclonal antibody against IL-1Ra and incubated on an orbital shaker for 2 hours. The plates were then washed four times with a non-phosphate containing buffer before IL-1Ra conjugated to horseradish peroxidase was added (200μL), before incubation and agitation for a further 2 hours. A further four washes were performed before substrate solution (hydrogen peroxide and tetramethylbenzidine with stabilisers; 200μL) was added to each well and the plate incubated for 20 minutes while protected from light while the colour developed. The reaction was stopped by the addition of stop solution (2N sulfuric acid; 50μL) and the plates immediately read in a microplate reader at 450nm (with wavelength correction at 570nm). Samples were compared to the standard curve and a final concentration obtained, which was averaged over the duplicates. The manufacturer's lower limit of detection of this assay was 14pg/mL, with an interassay CV of 4.4% at 918pg/mL and 5.0% at 1851pg/mL.

#### *2.6.3.2.3 Neopterin*

Assays were performed using pre-packaged kits (Brahms, Hennigsdorf, Germany) with all samples being studied in duplicate in a 96-well plate according to the manufacturer's instructions. Enzyme conjugate (neopterin/alkaline phosphatase; 150μL) and citrated plasma samples, standards (2-250nmol/L) or known controls (50μL) were added to a 96 well clean plate and mixed. A portion (150μL) of this

was transferred to a second plate coated with polyclonal sheep anti-neopterin antibodies and incubated in the dark for 2 hours. The enzyme conjugate and neopterin in the plasma samples compete for binding to the fixed antibody. Following incubation the plate was washed four times to remove all unbound components and 4-nitrophenyl phosphate (PNP; 100µL) was added to each well. Bound alkaline phosphatase conjugate catalyses the cleavage of the phosphate from PNP to produce the yellow 4-nitrophenol. The intensity of the colour is inversely proportional to the amount of neopterin in the plasma. The reaction was stopped by the addition of sodium hydroxide (100µL) and optical density measured in a microplate photometer at 405nm (with correction at 630nm). Samples were compared to the standard curve and a final concentration obtained, which was averaged over the duplicates. Accuracy of the assay was determined with reference to the known high and low concentration control samples. The manufacturer's lower limit of detection of this assay was 2nmol/L, with an interassay CV of 9.6% at 7.19nmol/L and 5.14% at 65.42nmol/L.

#### *2.6.3.2.4 C-reactive protein*

Plasma C-reactive protein values in the *in vivo* study were determined, with a standard laboratory technique, using a high sensitivity automated microparticle enhanced latex turbidimetric immunoassay (COBAS MIRA; Roche Diagnostics GmbH)([460]). Serum collected in lithium heparin, as described previously (section 2.6.2), was recentrifuged at 4000G for 10 minutes in a chilled centrifuge and the supernatant removed. A blinded operator measured samples in batches. The lower limit of detection of this assay was 0.2 mg/l, with a CV of 4.2% at 4 mg/l

and 6.3% at 1 mg/l. The assay was standardised with the appropriate WHO reference standard([461]).

#### *2.6.3.2.5 Tumour necrosis factor α*

Assays were performed using a pre-packaged kit (R and D Systems, Abingdon, Oxon) with all samples being studied in duplicate in a 96-well plate according to the manufacturer's instructions. Assay diluent (50μL) and standards (over a range of 0.5 to 32pg/mL) or citrated plasma samples (200μL) were added to the plates coated with mouse monoclonal antibody against TNFα and incubated on an orbital shaker for 3 hours. The plates were then washed six times with a non-phosphate containing buffer before TNFα conjugated to alkaline phosphatase was added (200μL), before incubation and agitation for a further 2 hours. A further six washes were performed before substrate solution (NADPH with stabilisers; 50μL) was added to each well and the plate incubated for 60 minutes on the desktop. Amplification was performed for 30 minutes after addition of the relevant amplifier solution (alcohol dehydrogenase and diaphorase; 50μL). NADH, formed from NADPH by reaction with the fixed alkaline phosphatase in a dose dependent fashion, reduced a tetrazolium salt to produce a coloured formazan dye (and NAD+ then is recycled in the amplification step). The reaction was stopped by the addition of stop solution (2N sulfuric acid; 50μL) and the plates immediately read in a microplate reader at 490nm (with wavelength correction at 690nm). Samples were compared to the standard curve and a final concentration obtained, which was averaged over the duplicates. The manufacturer's lower limit of detection of this assay was 0.12pg/mL, with an interassay CV of 12.6% at 6.7pg/mL and 10.8% at 13.5pg/mL.

### 2.6.4 Biochemical assessment of oxidant stress

A pro-oxidant environment can reduce NO bioavailability by directly breaking down the NO or oxidising $BH_4$ as discussed in the introduction (section 1.10.1.2). This section describes the assays carried out to determine changes in the blood oxidant profile and concentrations of reduced ascorbic acid before and after vaccination.

#### 2.6.4.1 Total Anti-Oxidant Status

These assays were performed in a blinded fashion with the assistance of Dr Jeffrey Stephens, who has validated this assay([462]). Plasma Total Anti-Oxidant Status (TAOS) was measured with a photometric assay initially described by Laight *et al* and modified by Sampson et al([413;463]). The assay depends on the capacity of plasma to inhibit the peroxidase-mediated formation of the 2,2-azino-bis-3-ethylbensthiazoline-6-sulfonic acid ($ABTS^+$) radical and is compared to the amount of $ABTS^+$ produced when phosphate buffered saline (PBS) is used as a control. The effects are detected by the colour change of horseradish peroxidase induced in a quantitative fashion by the $ABTS^+$.

Although this technique is used as a non-specific assay of multiple anti-oxidant pathways within the blood the activity of catalase may be most important component. In support of its use in these studies TAOS has been found to be closely correlated with other markers of oxidant stress, in particular F2-isoprostanes([464]).

The assay was performed in a clean 96 well plate with all samples analysed in duplicate. Stock solutions were made prior to the assay. Plasma samples or PBS controls (2.5µl) are added to all bar two wells, which are used as blanks, with two known positive controls. To each well 20µl ABTS (20mmol/L; Sigma), 20µl horseradish peroxidase (30mU/ml; Merck) and 37.5µl PBS was added prior to commencing the reaction with 20µl hydrogen peroxide (final concentration 0.1mmol/L; Sigma). The reaction proceeded in the dark at $37^0$C for 10 minutes and was read at 405nm using a Labsystems Multiscan Ex microplate reader. The difference in absorbance (control absorbance minus test absorbance) divided by the control absorbance (expressed as a percentage) represents the degree of inhibition of the reaction, and therefore the total anti-oxidant status of the plasma samples.

### 2.6.4.2 Acorbic Acid concentration

Dr Chrissi Dunster at King's College London performed these assays for this work in a blinded fashion on samples collected by myself. Venous samples were collected onto ice and centrifuged at $4^0$C (3000G for 10 minutes) before being mixed with equal volumes of chilled fresh 10% metaphosphoric acid and recentrifuged. The supernatant was drawn off and stored at $-20^0$C and then transported to King's College for further analysis.

Samples were defrosted and diluted 5:1 with metaphosphoric acid to produce a final volume of 1mL and mixed. Heptane (200µL) was added and vortexed before centrifuging at $4^0$C for 5 minutes (13000rpm). The supernatant was collected and diluted with more heptane before repeated centrifugation, on two

further occasions. The ascorbic acid concentration was determined with High Performance Liquid Chromatography (HPLC).

The HPLC was run with the following parameters:

Mobile phase = 0.2M $KH_2PO_4$ / 0.25mM octane sulphonic acid, pH2.1 at 1ml/min

Column = 250 x 4.6mm ApexII ODS 5micron column with 2cm Bio300 guard

Glassy carbon electrode = 400 - 600mV potential for measurement of vitamin C.

Range = 0.5 – 1.0 μamps sensitivity

Samples were compared to standards made with ascorbic and uric acid dissolved in metaphosphoric acid at concentrations of 0, 3.125, 6.25, 12.5, 25, 50μM on each occasion.

### 2.6.5 Determination of NO production by Greiss assay

The Griess assay is a colorimetric technique for detecting nitrite concentrations by the azo coupling of diazonium, which is itself produced by the reaction of nitrite with sulphanilamide, with naphthylethylenediamine (NED). This produces a purple dye (wavelength 540nm) in a quantitative manner that is then detected with a plate colourimeter. In these experiments, in the interests of simplicity, no attempt was made to reduce nitrate to nitrite and therefore only the latter was measured.

A series of seven standards were produced in DMEM from 2-100μM of nitrite from a known $10^{-3}$M stock solution. These were added in duplicate (100μl per well) to the first three columns of wells on each plate with the last row being used

for DMEM alone. The two Griess reagents were made; "A" consisting of 5% phosphoric acid and 1% sulphanilamide and "B" of 0.1% NED. These were mixed in equal volumes and 100µl of the resultant solution added to each well. The plate was allowed to incubate on the desktop and then the absorbance read (at 540nm) on a photometer. A standard curve was produced and the raw data converted into concentrations of nitrite by reference to this, having subtracted the effect of the DMEM blank. All results were then corrected for the protein concentration (producing a result as µM nitrite per µg protein) and averaged over the four identical treatments on each experiment.

### 2.6.6 Determination of pH by colourimetric assay

As DMEM contains the pH dependent dye phenol red it is possible to see changes in the pH of the solution by changes in this dye (from yellow for acidic to pink for alkali). In these experiments pH was determined by two methods, firstly the use of pH sensitive paper strips and secondly by taking advantage of the colour change to perform a colourimetric test.

The strips (pH-FIX; Sigma, UK) were chosen for their range of 6-9 units and 50µl of solution was added to each. The result was read from the colour standard provided with the strips.

In order to more accurately quantify the results a standard curve of 8 different pHs was generated over the range of 6-9 by the addition of increasing quantities of 0.25M hydrochloric acid to a stock solution of DMEM. Initially the pH of the solution was determined with a pH meter (Hanna Instruments pH211 meter,

Leighton Buzzard, UK) and 100µl added in duplicate to the 96 well plate with the culture media samples. Hydrochloric acid was then added and the pH redetermined, aiming to produce a 0.5 unit fall in pH each time, and the resultant solution aliquoted into the 96 well plate. This was repeated to produce the standard curve and then the plate was read with a photometer (wavelength 595nm).

Unknown samples were then transformed into pH by reference to the standard curve and averaged over the four identical treatments.

## 2.7 Data analysis

### 2.7.1 Data handling

#### 2.7.1.1 *In vitro* studies

In organ bath studies concentration-response curves for vasoconstrictors were generated by firstly subtracting the baseline tone of the vessels (approximately 1g for rat and 2g for human tissue) from the tone at each time point. Results were then plotted as active tone against drug concentration (on a log10 scale) using the mean (± standard error of the mean (SEM)) for the multiple replicates of each experiment. In the case of vasorelaxants, which were initially pre-constricted with phenylephrine, the results were expressed as percentage relaxation from the maximal constriction after subtraction of the basal tone in all cases. Again data was plotted as mean (±SEM).

Protein intensity blots were semi-quantified by intensity and expressed as mean (±SEM) over at least three groups. Other biochemical parameters were also expressed as mean (±SEM).

### 2.7.1.2 *In vivo* studies

In studies using plethysmography the slope of the steepest part of the MacLab recording from the strain gauges was determined for both arms for the last 4 recordings of each dose of agent and baseline. Although this gives actual flow (in mL/100mL forearm/min) a ratio of active to inactive arm was calculated, to allow the inactive arm to control for changes in ambient conditions. The percentage change in the averaged active ratio compared to the baseline ratio was determined and this was plotted in each case. In addition the area under the curve for each individual dose-response was calculated and also plotted. In all cases the data was expressed as means (±SEM). For studies involving the assessment of flow mediated dilatation the data was analysed with an automated system. The resultant percentage changes were displayed as means (±SEM). In the case of biochemical data parameters with a Gaussian distribution were expressed as mean (±SEM), while skewed data was shown as medians with 95% confidence intervals or geometric mean (± approximate SD).

### 2.7.2 Statistical analysis

#### 2.7.2.1 Definition of significance

In all studies significance was defined as a α-value of less than 5%, indicating that the chance of the null hypothesis still being true even though the difference is greater than the critical value was less than 5%. In the case of studies where the means of more than two variables were compared by t-tests a Bonferroni correction was applied. This states that when 3 or more variables are considered the resultant "P value" was multiplied by all possible comparisons. For example, if 3 groups were used the outcome of A vs. B would be multiplied by 3 (as it would be possible to compare A:B, A:C and B:C) and only if the result was still less than 5% it would be considered statistically significant.

#### 2.7.2.2 *In vitro* models

In organ bath experiments with concentration-response curves, comparisons between treated and control vessels were made by 2-way ANOVA. For studies with only a single intervention, such as the effect of arginine on pre-constricted vessels, and in those involving intensity analysis comparison was made by paired t-test with post hoc correction if required.

#### 2.7.2.3 *In vivo* models

For plethysmography, comparison of dose-response curves was performed by 2-way ANOVA. Secondly the area under the curve was calculated and means then compared by paired t-tests. Data from FMD studies were compared by one-way

repeated measures ANOVA with post hoc Bonferroni analysis of all pairs of data. In the experiments with tetrahydrobiopterin the data was analysed by the repeated measures of covariance model (XTREG procedure in STATA8.2) to account for the change in basal flow caused by tetradydrobiopterin infusion. This analysis was performed by JP Casas within our group.

Normally distributed biochemical data were compared by paired t-tests, while non-parametric tests (Wilcoxon sign ranked test) were used for skewed data. For cytokine changes comparisons were performed using area under the curve transformations and a one-sample t test.

Correlation between changes in FMD and inflammatory markers and neopterin were made by linear regression, with appropriate log transformation of parameters that were not normally distributed.

All statistical tests were performed with commercially available software – GraphPad Prism (San Diego, California).

# 3 Human *in vivo* model of the time course of inflammation on vascular function

## 3.1 Background

An acute inflammatory insult leads to the synthesis and release of a variety of inflammatory and anti-inflammatory mediators, including interleukins and CRP. Epidemiological data indicates an association between the concentration of CRP or inflammatory cytokines and the risk of future atherothrombotic events([152;213;225]). Incident cardiovascular events have been shown to be predicted by the presence of endothelial dysfunction([97;98]). Cross-sectional studies have shown an inverse correlation between CRP concentration and endothelium-dependent vasodilatation([311;312]), but in these studies it has not been possible to divorce the raised CRP from the inflammatory milieu in which it exists. CRP may be causal; it may be a marker of other causal inflammatory products or may be elevated as result of endothelial dysfunction or the atherosclerotic risk factors that produce it.

Previous work by our group has shown that the acute inflammatory response induced by typhoid vaccination is followed by the development of transient, substantial endothelial dysfunction([324]). This model was discussed in section 2.3. This chapter describes experiments where this model was used to examine the temporal relationship between the vaccination, the circulating concentration of inflammatory mediators and the development of endothelial dysfunction.

## 3.2 Protocols

### 3.2.1 Experimental inflammation study

After obtaining approval from the University College London Research ethics committee, 12 healthy non-smoking male volunteers aged 28 (± 6) years who gave written informed consent were studied. Four subjects underwent a repeat assessment in the absence of an inflammatory stimulus.

| | |
|---|---|
| **Number of subjects** | 12 |
| **Age** (years) | 28 (6) |
| **Sex** | All male |
| **Systolic Blood pressure** (mmHg) | 121 (11) |
| **Diastolic Blood Pressure** (mmHg) | 73 (7) |
| **Body mass index** ($kg/m^2$) | 25.2 (3.2) |
| **Medications** | Nil |

**Table 3.1 Baseline characteristics of subjects in experimental inflammation study (mean (SD))**

Endothelial function was studied, blood sampled and haemodynamic data collected at the same time on 4 consecutive days in 12 healthy, non-obese, subjects. Salmonella typhi capsular polysaccharide vaccine 0.025mg (Typhim Vi,

Pasteur Merieux MSD) was injected into the gluteus muscle at 8am on the morning of the second day of the study (section 2.3). Blood samples were taken at 4pm on each day and 8am and 12pm on day 2 and analysed for cytokine, CRP and neopterin responses as detailed in section 2.6. Endothelial function was measured by flow-mediated dilatation, as detailed in section 2.2.2, at 4pm on each of the four days.

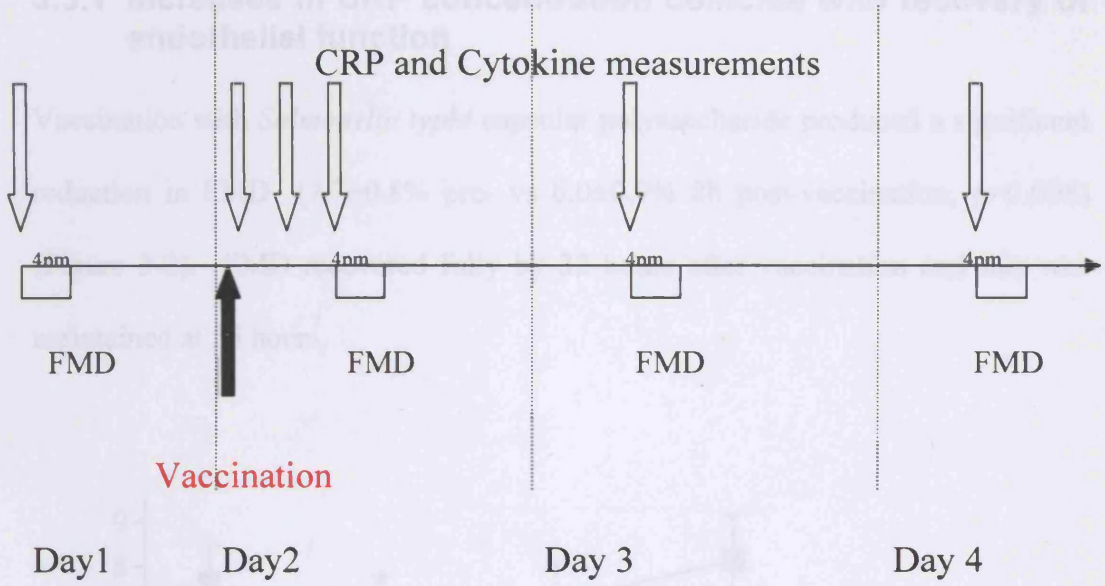



**Figure 3-1: Time line of study protocol. Arrows from above indicate blood sampling, arrow from below indicates vaccination.**

### 3.2.2 Statistical Analysis

Data are presented as mean and SEM unless otherwise stated. As they were not normally distributed, CRP concentrations were log transformed and were presented as geometric means and approximate standard deviations and heamodynamic parameters were expressed as medians with interquartile ranges. Comparison was made by repeated measures one-way ANOVA with a post hoc

Bonferroni analysis of all matched pairs. Correlations were performed between the changes in the CRP and IL-6 concentrations against changes in FMD and then a linear regression analysis. Significance was taken as $P<0.05$.

## 3.3 Results

### 3.3.1 Increases in CRP concentration coincide with recovery of endothelial function

Vaccination with *Salmonella typhi* capsular polysaccharide produced a significant reduction in FMD (7.7±0.8% pre- vs 6.0±0.9% 8h post-vaccination; p=0.008) (Figure 3-2). FMD recovered fully by 32 hours after vaccination and this was maintained at 56 hours.

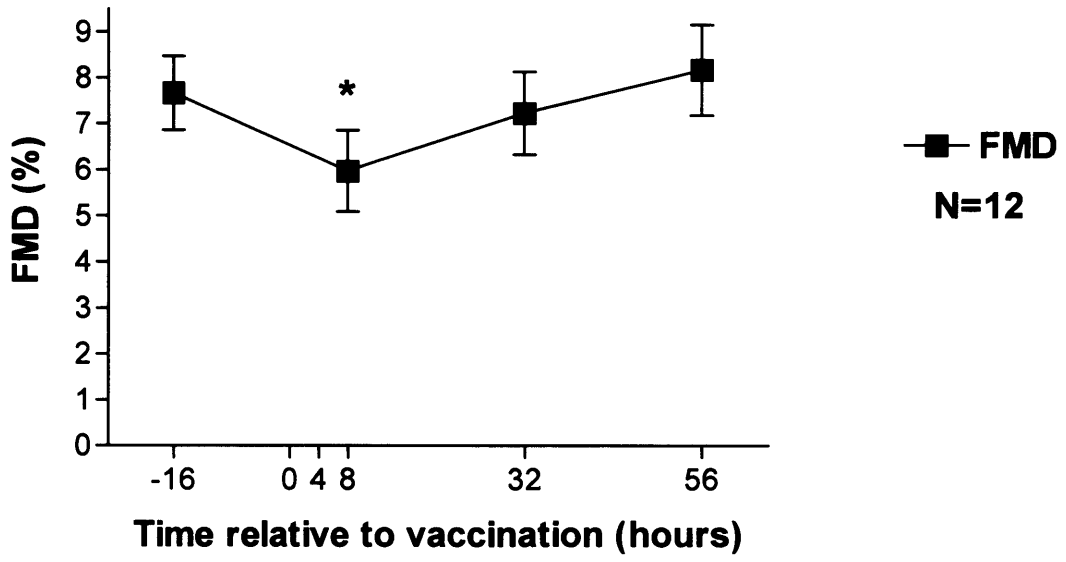



**Figure 3-2: Time course of endothelial response to vaccination. Significant reduction in FMD at 8 hours (P=0.01) relative to all other time points.**

At the time of maximal endothelial dysfunction, 8 hours after vaccination, CRP concentration was unchanged (0.89 [0.88] mg/L at baseline vs 1.06 [0.92] mg/L at 8h; p=0.3). However, by 32 hours after vaccination, CRP concentration was elevated 3-fold (to 2.7 [1.8] mg/L; p<0.01 vs baseline) (Figure 3-3) and this increase in CRP coincided temporally with the recovery of endothelial function (Figure 3-4).

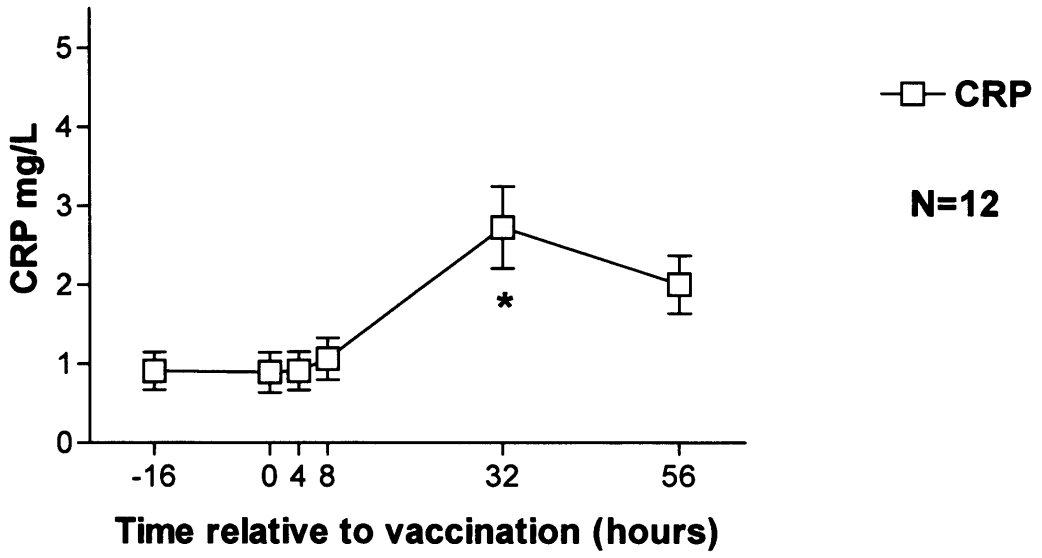



**Figure 3-3: Change in CRP concentration relative to time of vaccination. Significant rise in CRP at 32 hours relative to baseline (P=0.01 by post hoc analysis of repeated measures ANOVA).**

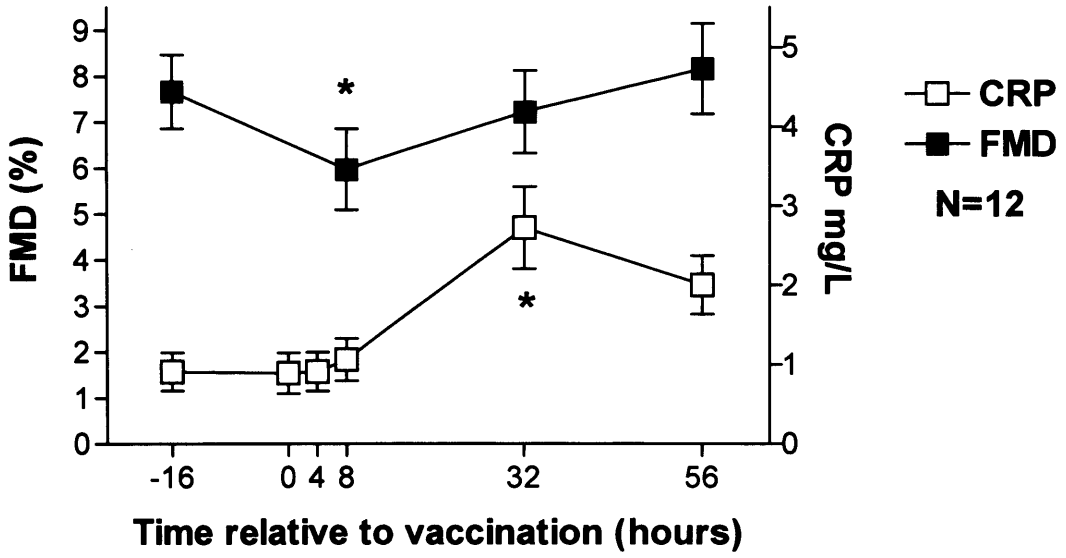



**Figure 3-4: Changes *in vivo* in flow mediated dilatation of the human brachial artery (■) and CRP (□) relative to Salmonella typhi vaccination. (N=12; P<0.01 by repeated measures ANOVA for both CRP and FMD).**

### 3.3.2 Changes in cytokine concentrations precede the development of endothelial dysfunction

In contrast, concentrations of IL-6 and IL-1Ra were both significantly elevated by 4 hours after vaccination, prior to the development of maximal endothelial dysfunction, and returned to baseline by 32 hours (IL-6 0.87±0.08pg/mL at baseline and 5.8±1.6pg/mL at 4 hours post-vaccination; and IL-1Ra 265±49pg/mL at baseline and 363±101pg/mL at 4 hours post-vaccination; $P<0.05$ for both comparisons) (Figure 3-5).

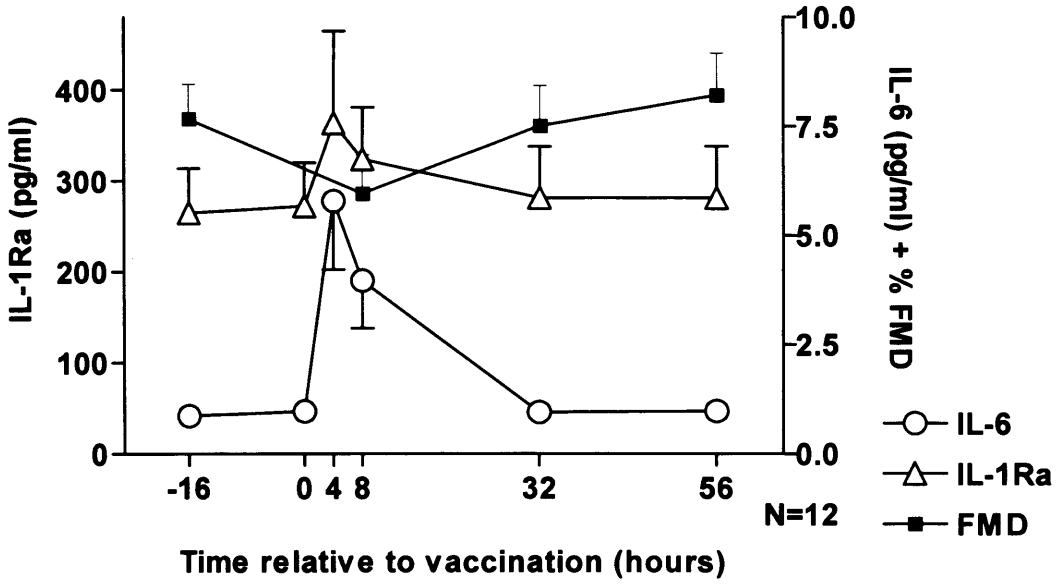



**Figure 3-5: Changes *in vivo* in flow mediated dilatation of the human brachial artery (■), IL-6 (○) and IL-1Ra (Δ) relative to Salmonella typhi vaccination. (N=12; P<0.01 by one-way ANOVA for CRP and P<0.05 for IL-6 and IL-1Ra).**

### 3.3.3 Vaccination does not lead to an alteration in haemodynamic parameters or the response to GTN

Baseline vessel diameter, response to GTN and temperature, pulse and blood pressure did not change over the study period (Figures 3-6 and 3-7 respectively). The degree of reactive hyperaemia also did not alter at different time points (Figure 3-8). Neither CRP nor FMD varied during the time control studies (P=0.6 and 0.5 respectively by repeated measures ANOVA) (Figure 3-9).

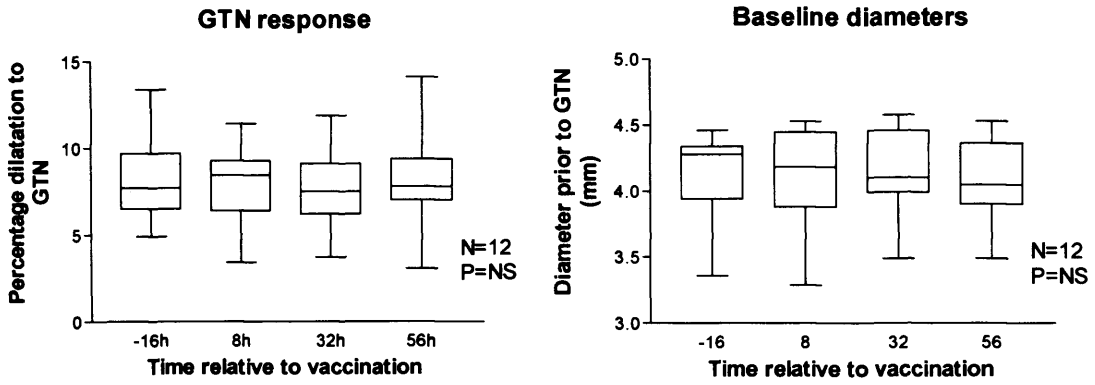



**Figure 3-6: Baseline vessel diameters for FMD analysis and the response to the control vasodilator GTN are not changed by vaccination.**

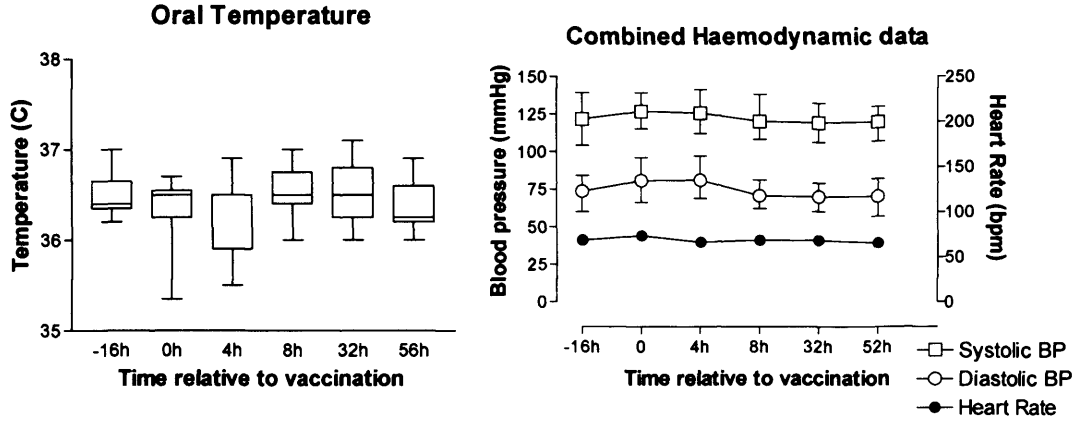



**Figure 3-7: Haemodynamic changes relative to vaccination. No significant change in temperature, pulse or blood pressure.**

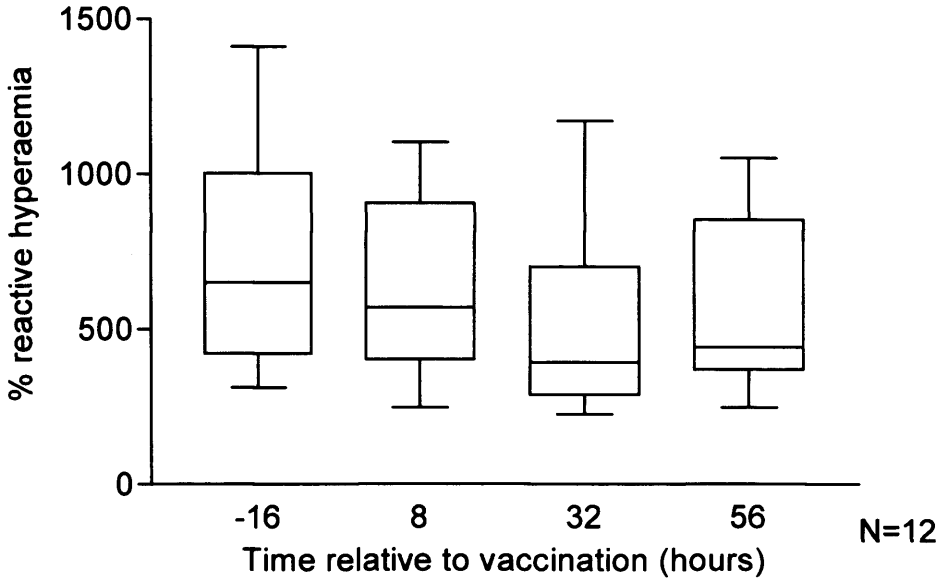



**Figure 3-8: Hyperaemic response does not alter significantly at different time points (P=0.3 by repeated measures ANOVA).**

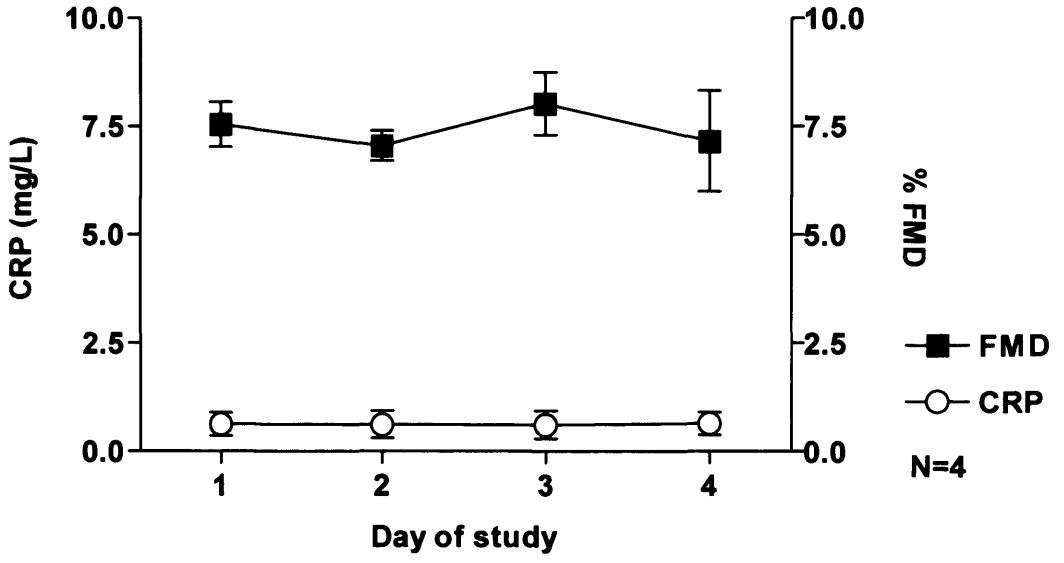



**Figure 3-9: Time control study. CRP and FMD do not alter significantly over time in the absence of vaccination (P=0.5 for FMD and P=0.6 for CRP)**

### 3.3.4 IL-6, but not CRP, concentrations correlate with FMD responses

Analysis, of all the time points at which endothelial function was assessed, of the change in FMD from the proceeding day compared to the change in serum markers showed no correlation between CRP and FMD (N=36, $r^2$=0.02, P=0.45) but an inverse relationship between IL-6 and FMD (N=36, $r^2$=0.36, P<0.001) (Figure 3-10).

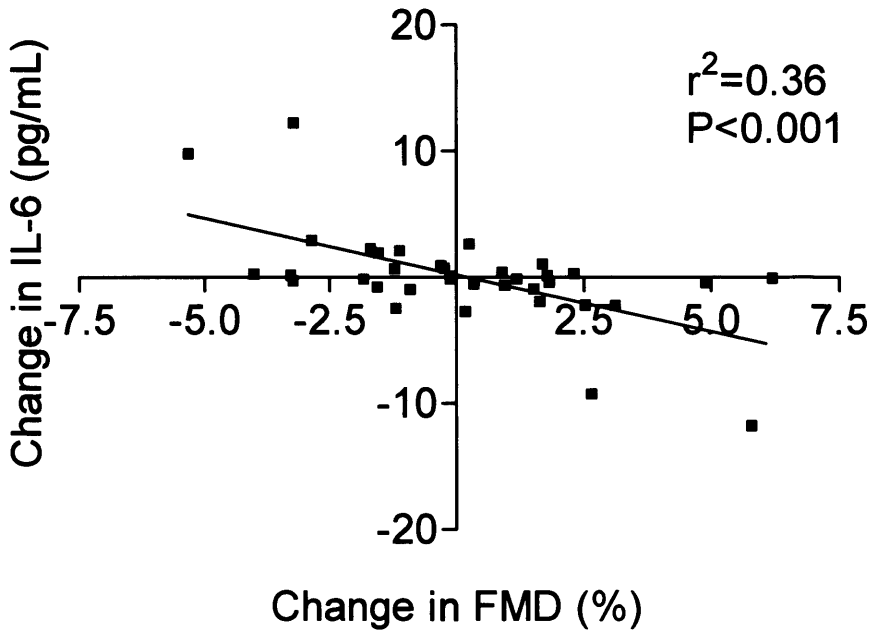



**Figure 3-10: Linear regression between the changes in endothelial function compared to the changes in IL-6 concentrations between each day of the study. (N=36, $r^2$=0.365, P<0.001)**

## 3.4 Discussion

CRP is well characterised as an acute phase reactant that may have an important role in host defence against infection. Measurement of CRP concentrations within the range found in health has been shown to be strongly predictive of future

cardiovascular events. This has led to the proposal that CRP itself might play a role in atherogenesis, a proposal supported by data from a small number of studies that have described associations between CRP and the presence of endothelial dysfunction([311;312]).

Work by other authors, conducted *in vitro*, suggests that CRP causes a reduction in nitric oxide bioavailability and would therefore be expected to be associated with endothelial dysfunction([298;299]). This was studied in this model of inflammatory endothelial dysfunction in humans, in which impaired FMD of the brachial artery is observed 8 hours after intra-muscular administration of *Salmonella typhi* capsular polysaccharide vaccine. The reduction in FMD was preceded by increases in IL-6 and IL-1Ra. Using a highly sensitive assay, no detectable changes in CRP concentration were found prior to the development of endothelial dysfunction. However, CRP values were significantly increased following this time point (from 1.5mg/L [0.4-2.0mg/L] at 8 hours to 2.8mg/L [1.9-3.8mg/L] at 32 hours; $p<0.001$) and were associated temporally with recovery, rather than further impairment, of endothelial function. Since FMD of the brachial artery, following this short hyperaemic response, is almost exclusively dependent on the release of endothelial NO, changes in FMD were taken to reflect alterations in NO bioavailability([452;465]).

Although vaccination did lead to a change in endothelial function this was not accompanied by an alteration in haemodynamic parameters. Importantly there was not a significant change in the hyperaemic response following cuff inflation, measured by the velocity-time integral, which could have confounded the results.

In fact there was a trend to a towards a smaller hyperaemic response at 32 hours that would if anything lead to an underestimation of the FMD at this point. As has been shown previously([324]), there was no alteration in blood pressure with vaccination. This may be because the vessels studied were conduit and not resistance structures and therefore any changes seen may have a predominant effect in these arteries, or that the increased NO generation leads to acute reflex compensatory mechanisms that serve to maintain blood pressure.

In previous cross-sectional studies conducted *in vivo*, CRP concentrations have been shown to correlate inversely with NO-mediated endothelium-dependent vasodilation in patients with, or at-risk of, atherosclerosis([311;312]), and this has been taken as evidence that CRP causes endothelial dysfunction. However, the correlation between CRP and endothelial function in cross-sectional studies is likely to be confounded by the presence in the circulation of elevated concentrations of cytokines and other pro-inflammatory factors, and does not therefore prove a causal link.

In this study differences in the time-course of induction of inflammatory cytokines and CRP revealed a temporal dissociation between the development of endothelial dysfunction and the induction of CRP synthesis. Following a sterile inflammatory stimulus induced by *S.typhi* capsular polysaccharide vaccination, increases in circulating CRP concentration occurred after the development of endothelial dysfunction which was preceded by increases in IL-1Ra (a marker of IL-1 activation) and IL-6. It is possible, therefore, that IL-1 or IL-6, or their downstream effectors contribute to the development of endothelial dysfunction,

while the later elevation of CRP plays a part in its resolution. These results are further supported by the observation that changes in IL-6 concentrations, but not CRP, are correlated with changes in FMD when the entire time course is considered.

Despite these likely explanations for the differences between our study and previous reports, we cannot completely discount the possibility that CRP has pleiotropic effects on the vasculature, that differ according to the time course of exposure, concentration achieved and the prevailing cytokine milieu. It is not possible to infer causality in the association between CRP concentration and changes in FMD, as it is possible that in the absence of a rise in CRP on the third day that the FMD response may have been even more marked and therefore the CRP is still acting to suppress NO release.

In summary, this study suggests that IL-6 and IL-1Ra could be involved in the processes that lead to endothelial dysfunction following typhoid vaccination, but that CRP changes are too slow to be causal. This thesis will now go on to explore the mechanisms by which inflammation leads to endothelial dysfunction and to further explore, *in vivo,* the mechanisms by which CRP might lead to improvements in endothelial function.

# 4 Determination of the effect of experimental inflammation upon the nitric oxide pathway *in vivo*

## 4.1 Background

The healthy vascular endothelium has vasodilator, anti-adhesive, anti-inflammatory and anti-coagulant properties, through the production of mediators, including nitric oxide (NO). Endothelial dysfunction is an early event in the pathogenesis of atherosclerosis([108;466]) and is characterised by reduced dilator function, increased inflammatory cell and platelet adhesion([467]), and increased coagulant activity([468]). Reduced bioavailability of NO makes a major contribution to endothelial dysfunction and may be the result of reduced NO synthesis, due to substrate or co-factor deficiency, or increased NO breakdown, due to chemical reaction with oxidant radicals.

One potential trigger for endothelial dysfunction is inflammation([23;149-151;469;470]). Inflammatory cytokines impair endothelial function in animal models([471]) and isolated human veins([322]). Previous work by our group has shown that acute systemic inflammation induced by vaccination leads to transient endothelial dysfunction, characterised by reduced response to the endothelium-dependent vasodilators bradykinin and acetylcholine in the forearm arterial bed([324]). However, the precise mechanism of this effect has not been previously explored.

The aims of this chapter were to determine whether this experimental inflammatory stimulus also alters basal NO-mediated dilatation, to determine the

mechanisms of the effects seen and to explore whether endothelial dysfunction is evident systemically.

## 4.2 Protocols

### 4.2.1 Subjects

Twenty-one male and female subjects aged 22 to 40 were studied. No subjects had received typhoid vaccination in the preceding 6 months. Individuals were studied at the same time of day on two consecutive afternoons.

| | |
|---|---|
| **Number of Subjects** | 21 |
| **Age** (years) | 29 (6) |
| **Sex** (male/female) | 15/6 |
| **Systolic Blood Pressure** (mmHg) | 122 (12) |
| **Diastolic Blood Pressure** (mmHg) | 72 (6) |
| **Medications** | Nil |

**Table 4.1 Baseline characteristics of subjects in these studies (mean (SD))**

### 4.2.2 Generation of an inflammatory response

Salmonella typhi capsular polysaccharide vaccine 0.025mg (Typhim Vi, Pasteur Merieux MSD) was injected into the gluteus muscle at 8am on the morning of the second day of the study (section 2.3).

### 4.2.3 Measurement of cytokines, serum total anti-oxidant status, serum albumin and lipids and urinary protein

Blood and urine samples were taken before and 2 hourly for 8 hours after vaccination for the measurement of serum albumin, plasma lipids and urinary protein. As a control in 8 subjects, urine samples were taken at 4pm on a different day in the absence of vaccination. In five subjects the plasma was assayed for interleukin-6 (IL-6) and interleukin-1 receptor antagonist (IL-1Ra). Plasma from 20 subjects (one was unsuitable) was assessed for total anti-oxidant status (TAOS). Urine was collected in sterile containers and frozen at –20°C immediately. Samples from only 17 subjects at baseline and 8 hours after vaccination were suitable (due to a storage failure) and therefore analysed for urinary albumin and creatinine concentrations. At a separate time samples were taken in 8 subjects at 8am and 4pm in the absence of vaccination to study urine albumin/creatinine ratios. Individual assays are described in section 2.6.

### 4.2.4 Assessment of forearm blood flow

Venous occlusion plethysmography was used to measure forearm blood flow 16 hours before vaccination (control) and 8 hours following vaccination as described in section 2.2.1.

#### 4.2.4.1 Protocol 1; Effect of vaccination on NO-mediated dilatation

In five individuals, forearm blood flow in response to intra-arterial infusion of the vasodilators bradykinin (BK; 20, 40, 80pmol/min; each dose for 3 minutes) and glyceryl trinitrate (GTN; 8, 16 and 32 nmol/min; each dose for 3 minutes) and vasoconstrictors $N^G$-monomethyl-L-arginine (L-NMMA; 1, 2, 4 μmol/min; each dose for 5 minutes) and norepinephrine (NE; 60, 120 and 240 pmol/min min; each dose for 5 minutes) were assessed before and after vaccination. The order of the infusions was randomised although, due to its long duration of action, L-NMMA was always infused last. Saline was infused for 15 minutes between drug infusions to allow restoration of baseline flow. In three subjects, time control studies were performed to determine variation in vasoconstrictor response over time. In these studies, forearm responses to L-NMMA and norepinephrine were determined 24 hours apart in the absence of typhoid vaccination.

#### 4.2.4.2 Protocol 2; Effect of L-arginine on endothelial function after vaccination

In five individuals, forearm blood flow in response to BK (20, 40, 80pmol/min; each dose for 3 minutes) and GTN (8, 16 and 32 nmol/min; each dose for 3 minutes) was assessed before and during co-infusion with L-arginine (50 μmol/min; preinfused for 15 minutes). This protocol was repeated before and after vaccination as above.

#### 4.2.4.3 Protocol 3; Effect of ascorbic acid on endothelial function after vaccination

In eight individuals, forearm blood flow in response to BK (20, 40, 80pmol/min; each dose for 3 minutes) and GTN (8, 16 and 32 nmol/min; each dose for 3 minutes) was assessed post-vaccination before and during co-infusion with ascorbic acid (25 mg/min; preinfused for 15 minutes).

### 4.2.5 Calculations and statistical analysis

The ratio of blood flow in the infused/non-infused (control) arm was calculated for each measurement period. Changes in flow were expressed as a percentage change in the ratio of forearm blood flow (infused/non-infused) relative to the immediately preceding baseline flow, as described in section 2.2.1. Results are expressed as mean ±SEM unless otherwise stated. Cumulative dose-response curves were constructed for all drugs and the area under the curve (AUC) calculated. Responses were compared by paired Student's t-test. The Wilcoxon sign ranked test was used for non-parametric data. The time course of the cytokine response was expressed as the AUC and analysed by a one-sample t-test. Analysis of the effect of L-arginine and ascorbic acid was carried out by assessment of the AUC for each dose response curve with paired t tests followed by the Bonferroni correction for multiple comparisons. $P<0.05$ was considered statistically significant.

### 4.2.6 Drugs

BK, NE and L-NMMA were obtained from Clinalfa AG; GTN from Faulding Pharmaceuticals and L-arginine and ascorbic acid from Medeva Pharma Ltd. All of the drugs were prepared as stock solutions and stored at –20°C until use.

## 4.3 Results

### 4.3.1 Systemic inflammatory response to vaccination

Two to four hours following vaccination there was a progressive rise in the IL-6 concentration from 1.6±0.27pg/mL at baseline to 5.6±1.3pg/mL at 8 hours (n=5, P=0.02). IL-1Ra levels rose after 6 hours from 186±42pg/mL at baseline to a peak of 332±78pg/mL before starting to fall, 8 hours following vaccination (n=5, P=0.02; Figure 4-1).

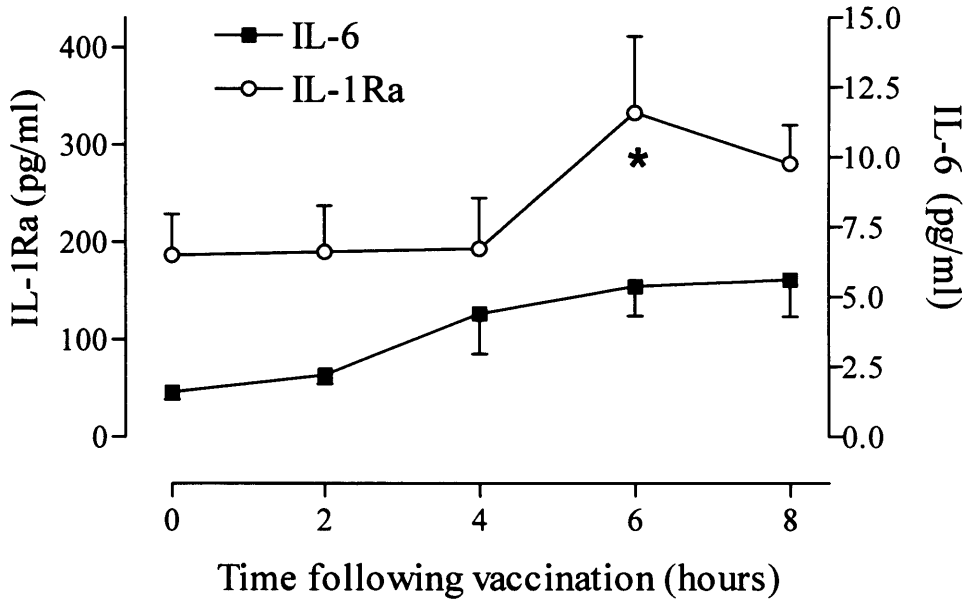



**Figure 4-1: IL-1Ra (○; left y axis) and IL-6 (■; right y axis) response in 8 hours following vaccination. There was a significant increase in the AUC for both cytokines (P=0.02)**

In comparison to baseline there was an increase in urinary albumin (p=0.03) and urinary albumin/creatinine ratios (UAC; p=0.05; Figure 4-2) at 8 hours following vaccination. In the absence of vaccination there was no diurnal variation in the UAC (8am 0.42±0.07, 4pm 0.45±0.09 mg albumin/mmol creatinine; p=NS; N=8).

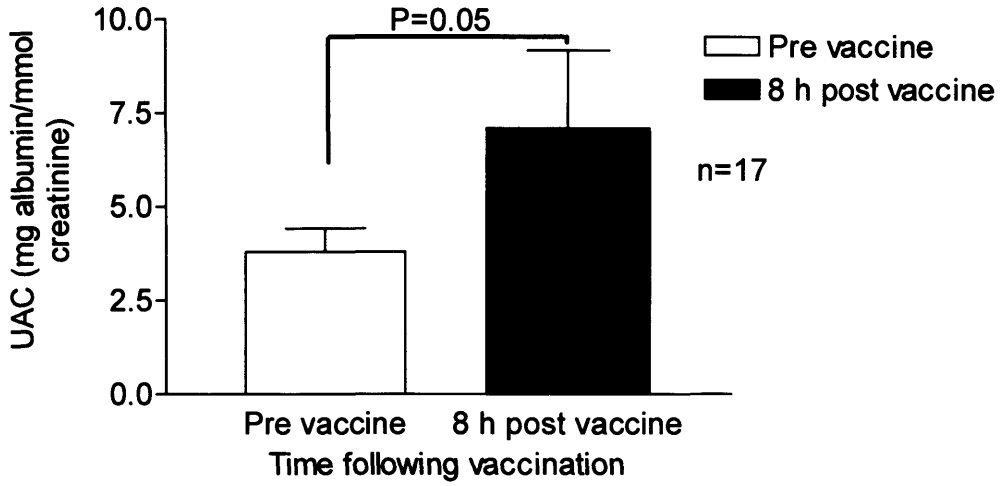



**Figure 4-2: Urinary albumin/creatinine ratio prior to and 8 hours following vaccination**

There were no significant changes in the serum levels of total (pre vaccine 4.56±0.15mmol/L, post vaccine 4.65±0.17mmol/L; p=0.07) or HDL cholesterol (pre vaccine 1.26±0.08mmol/L, post vaccine 1.28±0.09mmol/L; p=0.62) over this time course. Serum albumin increased following vaccination (42.5±0.5g/L pre-vaccine, 44.5±0.5g/L post-vaccine; p<0.01). TAOS decreased significantly eight hours following vaccination (33.7±3.7% at baseline to 21.3±3.4% at 8 hours; P=0.01; Figure 4-3) indicating an increase in oxidant stress at this time.

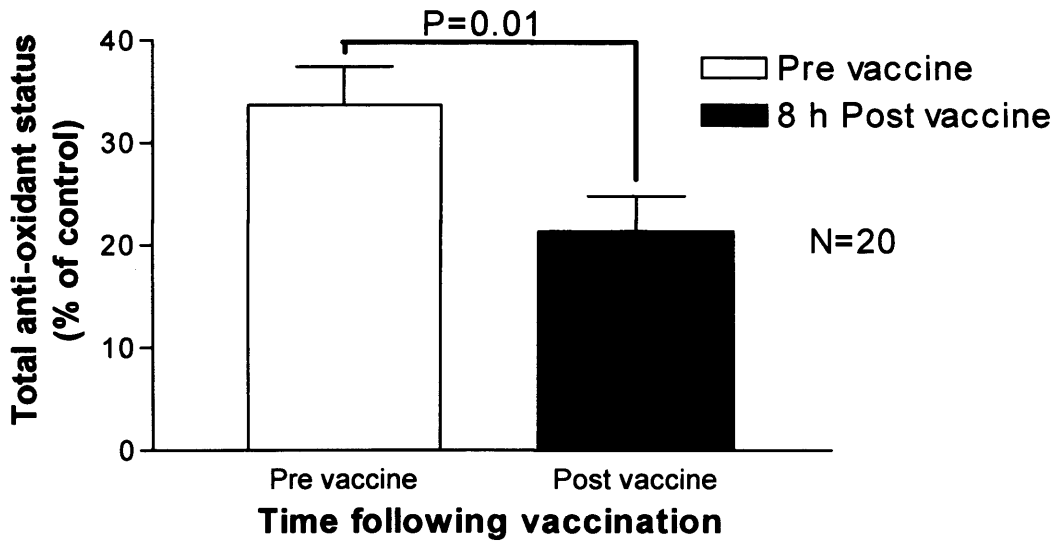
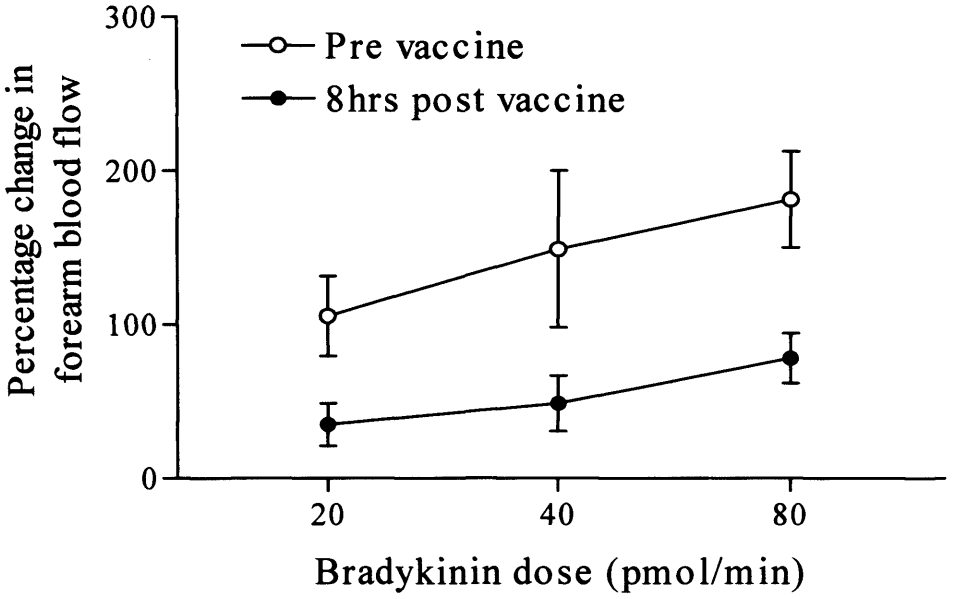



**Figure 4-3: Total anti-oxidant capacity of plasma prior to and 8 hours following vaccination expressed as a percentage of the saline control**

### 4.3.2 Forearm blood flow responses

Mean baseline blood flow did not change following vaccination (4.1±0.36ml/100ml forearm per minute before and 3.9±0.24ml/100ml forearm per minute 8 hours after). All subjects showed dose-dependent increases in blood flow in response to BK and GTN. Eight hours following vaccination the response to BK was significantly impaired (P=0.05 for AUC; Figure 4-4 A and B) while the response to GTN was unchanged (P=0.4 for AUC; Figure 4-5 A and B).

A




B

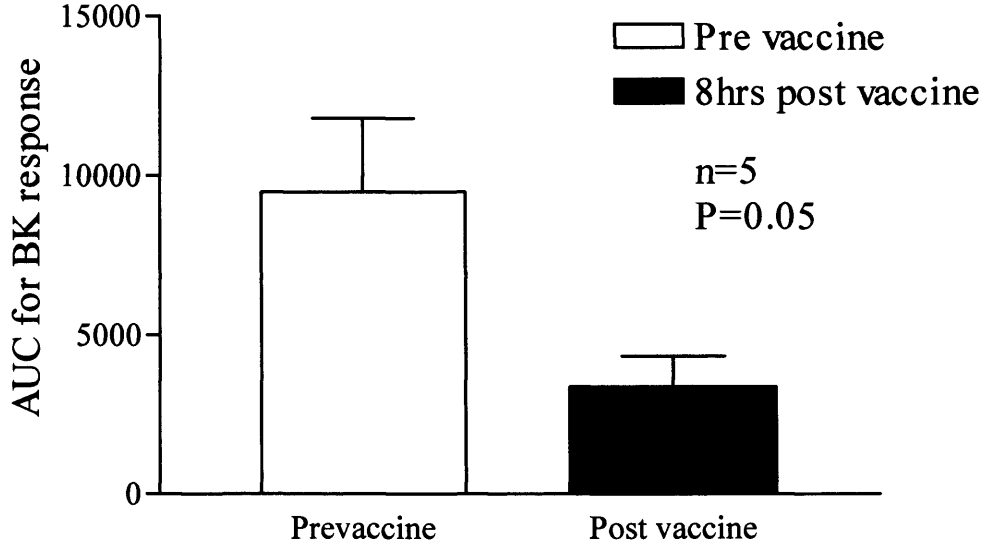



**Figure 4-4: Change in forearm blood flow in response to bradykinin before (○) and eight hours following vaccination (•) (A) and expressed as area under the curve (B) (n=5, P=0.05)**

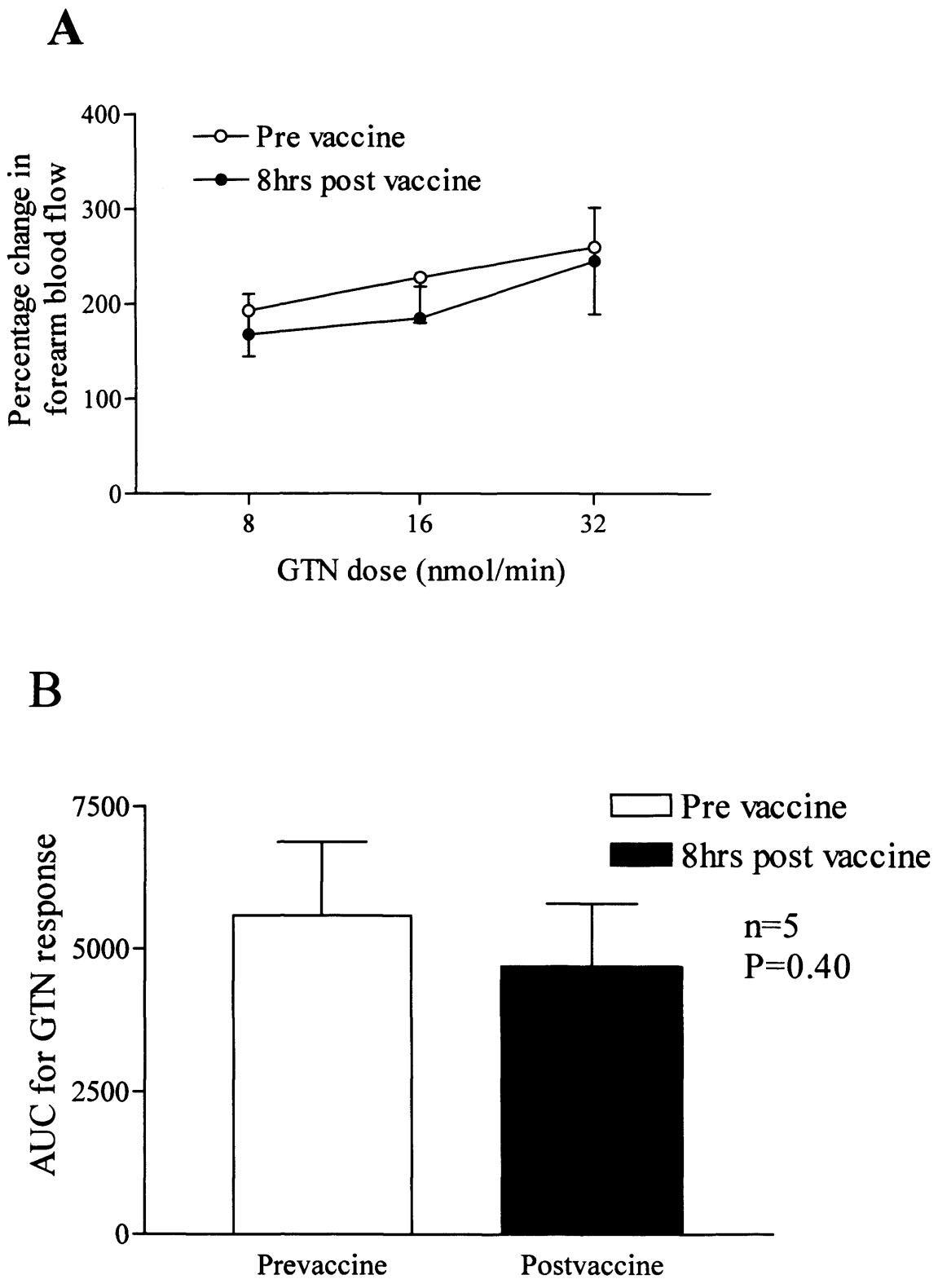
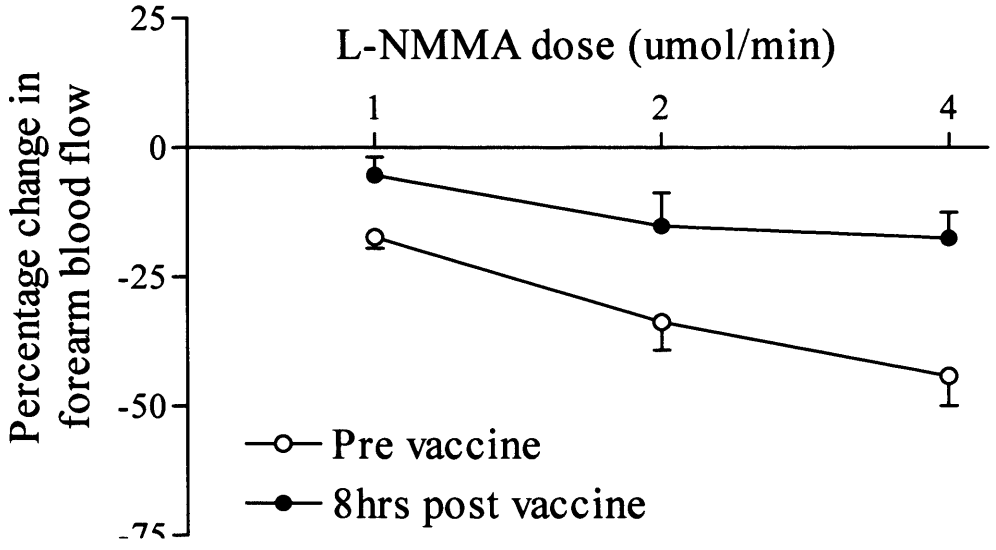



**Figure 4-5: Change in forearm blood flow in response to GTN before (○) and eight hours following vaccination (●) (A) and expressed as area under the curve (B) (n=5, P=NS)**

Prior to vaccination, L-NMMA and NE caused dose-dependent reductions in forearm blood flow. Eight hours following vaccination there was a reduction in the vasoconstriction to L-NMMA (P=0.02 for AUC; Figure 4-6 A and B) with no change in the response to NE (P=0.83 for AUC; Figures 4-7 A and B). In control studies, conducted in the absence of vaccination, there were no time-dependent changes in the response to L-NMMA (AUC: pre-vaccine 105 arbitrary units (AU), post-vaccine 125AU; P=0.6) or NE (AUC: pre-vaccine 6142AU, post-vaccine 3546AU; P=0.2) on consecutive days.

**A**



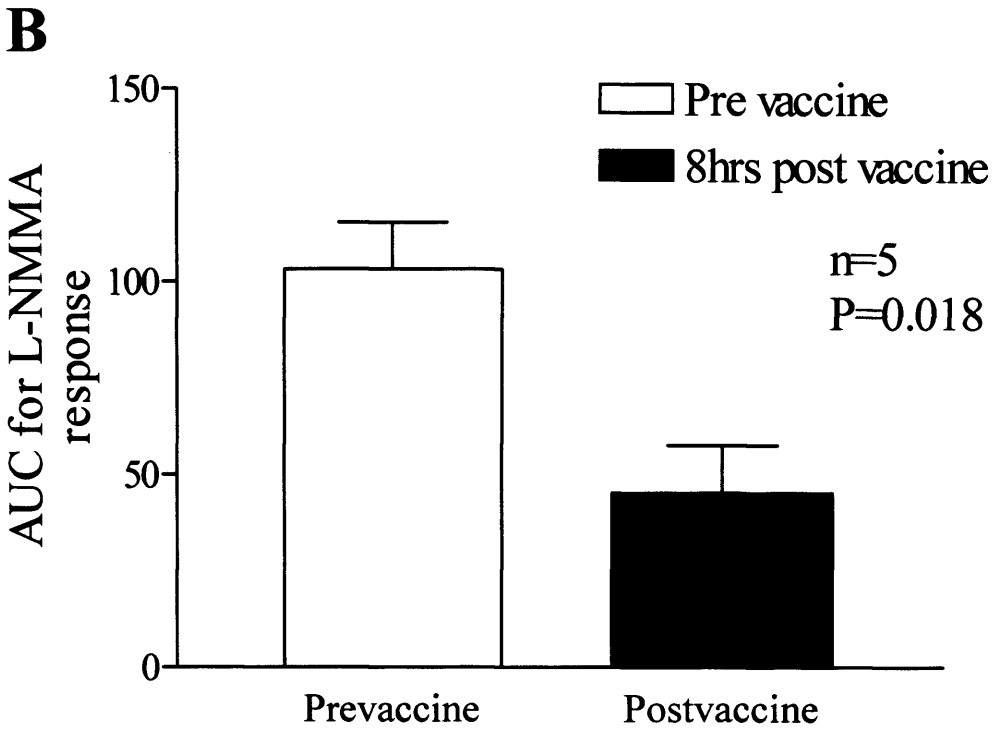
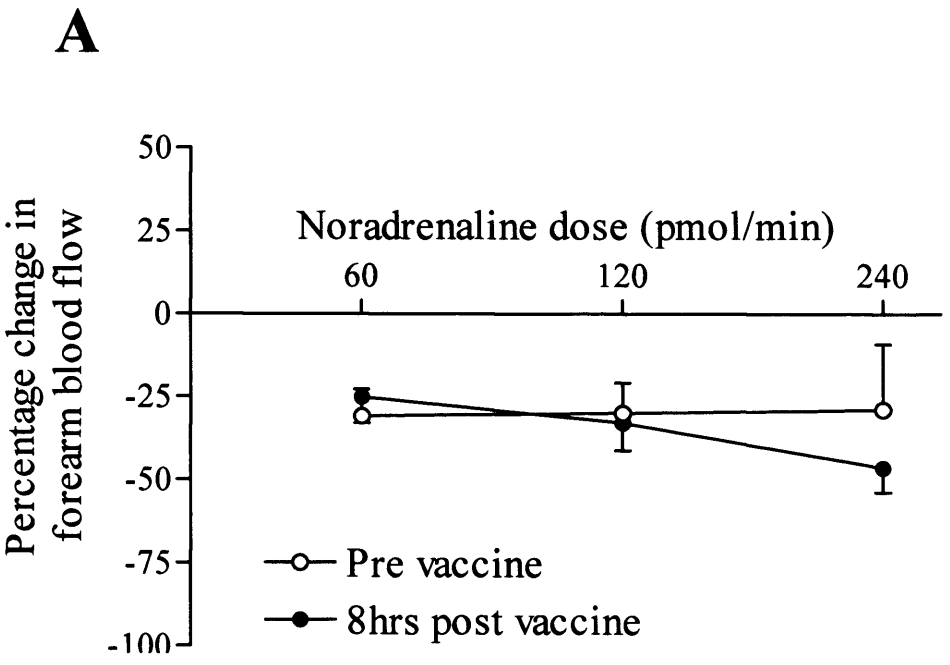



**Figure 4-6: Change in forearm blood flow in response to L-NMMA before (○) and eight hours following vaccination (●) (A) and expressed as area under the curve (B) (n=5, P=0.02 for AUC)**



**B**

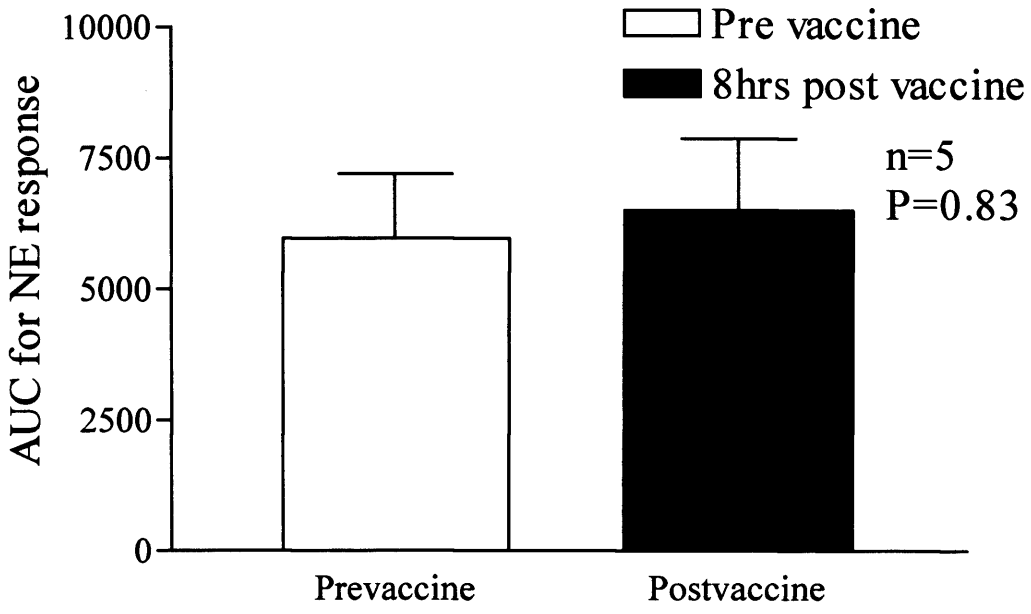



**Figure 4-7: Change in forearm blood flow in response to norepinephrine before (○) and eight hours following vaccination (●) (A) and expressed as area under the curve (B) (n=5, P=NS for AUC)**

Co-infusion of L-arginine (50μmol/min) had no effect on the response to bradykinin (Figure 4-8 A and B) or GTN before or after vaccination. However, infusion of ascorbic acid (25mg/min) after vaccination caused a partial restoration of the response to BK (AUC post vaccine 5004±1081 vs. post vaccine with ascorbic acid 7649±1124, P=0.04; Figure 4-9 A and B). Neither vaccination nor ascorbic acid had any effect upon the response to GTN (AUC pre-vaccine 3677±471, post-vaccine 4023±514 and with ascorbic acid 3158±370; P=NS)

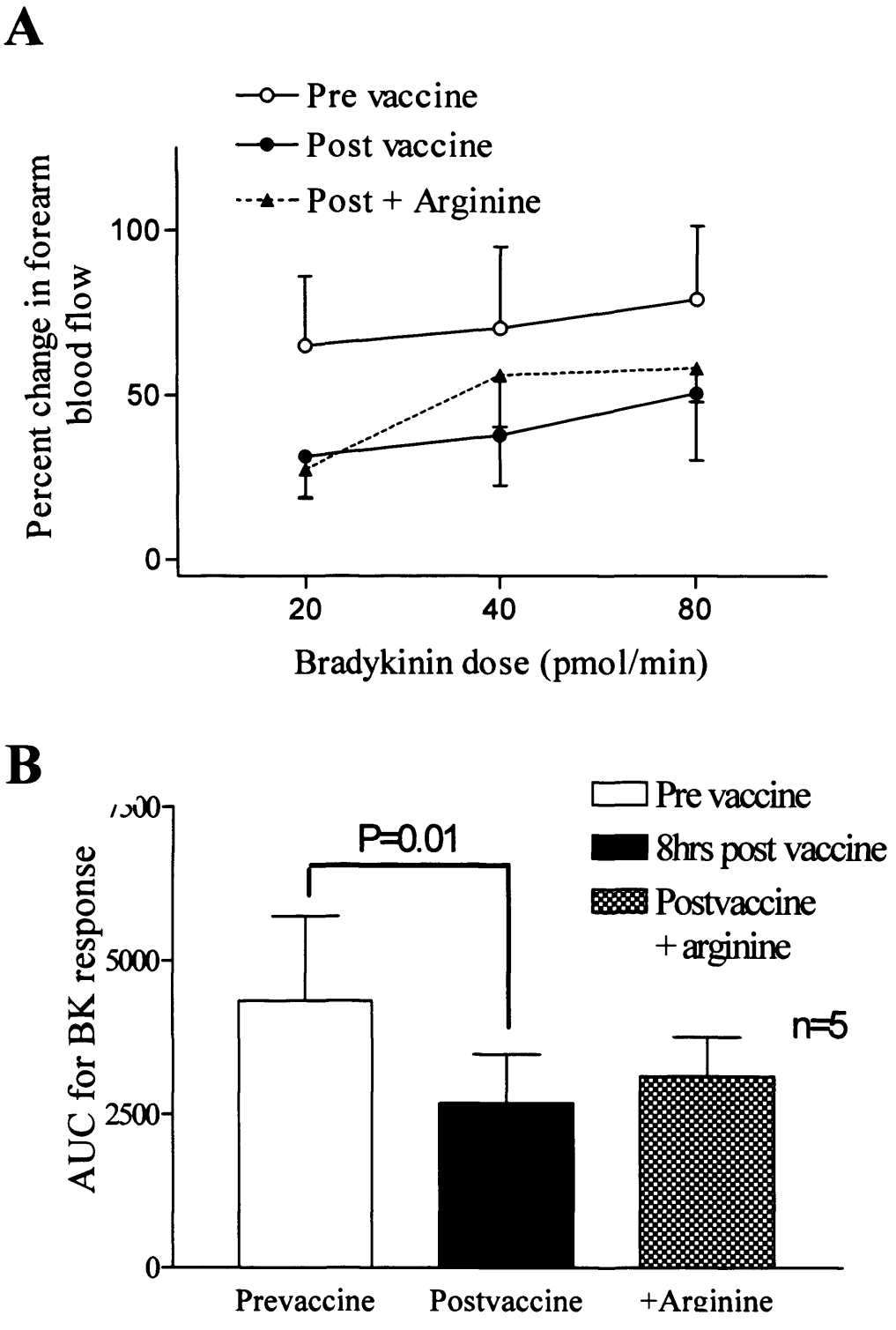



**Figure 4-8: Changes in forearm blood flow in response to bradykinin, expressed as dose response (A) and area under the curve (B), before and eight hours after vaccination with and without the addition of L-arginine (50µmol/min; n=5; P=NS for AUC). The response in the presence of arginine, prior to vaccination, is not shown.**

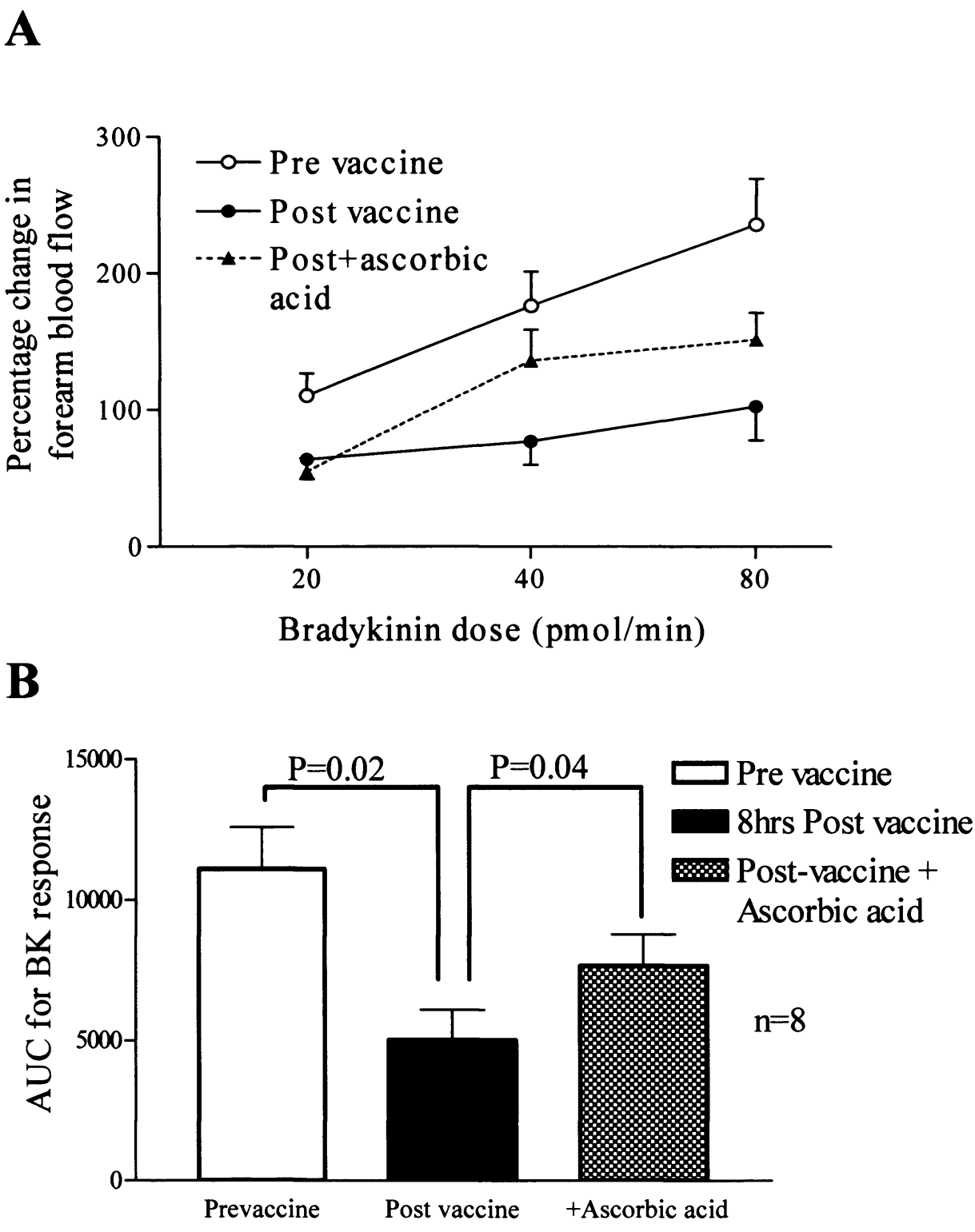



**Figure 4-9: Changes in forearm blood flow in response to bradykinin, expressed as dose response (A) and area under the curve (B), before vaccination and eight hours after vaccination with and without the addition of ascorbic acid (n=8; P=0.02 for pre-vaccine vs. post-vaccine, P=0.04 for post-vaccine with vs. without ascorbic acid)**

## 4.4 Discussion

These experiments confirm that inflammation caused by typhoid vaccination causes a pro-inflammatory cytokine response, and impairs forearm arterial endothelium-dependent dilatation. Vascular responses to L-NMMA were also diminished, indicating reduced basal NO bioavailability in the forearm ciculation. Urinary albumin excretion increased, which may be secondary to increased capillary permeability caused by endothelial dysfunction in the kidney and, by extrapolation, could indicate endothelial dysfunction in other organs and vascular beds. Despite the reduced NO bioactivity, supplementation with L-arginine 50μmol/min, the substrate for NO synthesis, did not restore endothelial function whereas infusion of the anti-oxidant ascorbic acid (25mg/min) led to a partial restoration of endothelium-dependent vasodilator responses. Plasma TAOS was decreased at the time of maximal endothelial dysfunction indicating an increase in oxidant stress. These findings implicate production of reactive oxygen species in the genesis of inflammation induced-endothelial dysfunction in humans.

In animals and humans, bradykinin vasodilates in part by an NO-dependent mechanism, with a probable contribution from endothelium-derived hyperpolarizing factor([57;472]). Previous work by our group has shown impaired bradykinin-induced vasodilatation following typhoid vaccination, with preservation of the response to the NO donor, GTN([50;324]). Administration of a high dose of aspirin before vaccination prevented inflammation-induced endothelial dysfunction, probably by blocking part of the cytokine response to vaccination([50]). In order to address specifically the role of the L-arginine:NO

pathway in vaccine induced endothelial dysfunction and to determine whether the protective basal NO mediated effects are lost, the vasoconstrictor response to L-NMMA was measured. The response to L-NMMA was markedly reduced following vaccination, with preservation of the response to the endothelium-independent vasoconstrictor norepinephrine. These data indicate that inflammation causes reduced activity of the basal endothelial dilator NO pathway. This may be biologically significant, because in animal models reduced NO bioavailability enhances atherogenesis and is associated with increased vascular resistance even if the blood pressure does not rise([473;474]).

Changes in NO bioavailability can be caused by a reduction in NO production or an increase in NO breakdown. Inhibition of NO synthesis with L-NMMA does not distinguish between these mechanisms. A reduction in smooth muscle sensitivity to NO was excluded given the normal vasodilatation observed to GTN not only in this study, but also in prior investigations([50;324]). Other potential mechanisms to account for reduced endogenous NO availability include depletion of the NOS substrate L-arginine or increased generation of reactive oxygen species, which combine with NO and lead to its breakdown, both of which have been reported in the context of endothelial dysfunction in the presence of orthodox cardiovascular risk factors.

Failure of L-arginine to restore the response to BK in the current study suggests that a deficiency of the substrate for NO synthase does not account for the effect of inflammation on the NO pathway. The dose of L-arginine administered is sufficient to raise L-arginine concentration into the low millimolar range([475]), and

previous work has shown that this dose of L-arginine improves endothelial dysfunction in the resistance vasculature of patients with hypercholesterolemia([476]), diabetes([115]) and heart failure([477]). Failure to respond to L-arginine supplementation suggests that the mechanism of endothelial dysfunction is not simply explained by acute L-arginine deficiency.

Ascorbic acid infused 8 hours following the inflammatory stimulus of vaccination partially restored the bradykinin response with no effect on the endothelium-independent vasodilator GTN. Ascorbic acid may exert antioxidant effects by direct quenching of reactive oxygen species([373]) or by stabilising the NOS cofactor tetrahydrobiopterin([379]). A significant fall in total anti-oxidant status of the plasma following vaccination may indicate an increase in free radical production with inflammation. However, concern has been raised as to which pathways are actually measured by this assay, and no specific assays of individual pathways were made to strengthen this association. That ascorbic acid partially reverses endothelial dysfunction supports a role for increased oxidant stress in inflammation-induced endothelial dysfunction. Similarly, ascorbic acid reversed endothelial dysfunction following administration of bacterial lipopolysaccharride([327]). Taken together with the observation that aspirin prevents endothelial dysfunction in this model([50]), it is possible that cyclo-oxygenase is a source of reactive oxygen species. Whether ascorbic acid acts to improve endothelial function by preventing oxidation of NO or promoting the stability of the $BH_4$ to increase NO generation([478]) remains to be determined. Failure to entirely restore the BK response may also reflect additional effects of

inflammation to reduce the activity of the NO pathway, including expressional changes in NOS.

Vaccination also caused increased UAC that was contemporaneous with changes in endothelial function and the rise in cytokines, and this may reflect transitory endothelial dysfunction in the kidney. In healthy volunteers there was a correlation between albuminuria and systemic endothelial function([479]), and increased urinary albumin excretion is commonly seen in clinical conditions where endothelial dysfunction is present, including diabetes mellitus([480]), hypertension([481]) cardiopulmonary bypass([482]) and sepsis([483]). Whether the microalbuminuria observed following typhoid vaccination can be explained by a transient reduction in renal NO production or represents a more generalised endothelial abnormality remains to be seen. Nevertheless, this combination of endothelial dysfunction, microalbuminuria, increased oxidant stress and a cytokine response suggests that typhoid vaccination causes the temporary development of a high cardiovascular risk phenotype. If this model accurately reflects the pathology of systemic inflammation then this may indicate a mechanism by which inflammatory events may lead to a transient rise in the incidence of cardiovascular events.

The measured lipid parameters did not change during these studies, indicating that this is not the mechanism by which inflammation induces endothelial dysfunction. However, it is possible that there were changes in the make-up of the different lipid fractions or modified lipids, for example an increase in oxidised LDL or small dense LDL, which might influence endothelial reactivity.

In summary, this study demonstrates that typhoid vaccination causes generalised endothelial dysfunction involving reduced NO bioavailability. This is not explained by substrate depletion but is partly dependent on increased oxidative stress. Naturally occurring infections and inflammatory stimuli may cause fluctuating endothelial dysfunction by similar means.

# 5 The role of anti-oxidants in the vascular response to inflammation

## 5.1 Background

The preceding two chapters have shown that inflammation, caused by typhoid vaccination, leads to a transient reduction in endothelial function. This was associated with an increase in serum oxidant stress and was partially reversible by ascorbic acid. This indicates that the changes observed may be due to increased oxidant breakdown of nitric oxide. However, the role of tetrahydrobiopterin bioavailability has not been directly addressed. This may be important, as local tetrahydrobiopterin infusion corrects endothelial dysfunction in established atherosclerosis([369]) or subjects at elevated cardiovascular risk([383;484]). Moreover, mice with targeted overexpression of GTPCH-1, the rate limiting enzyme in $BH_4$ synthesis, are protected from endothelial dysfunction that results from experimental diabetes([359]).

During the pilot studies for the previous work examining the effects of ascorbic acid on the impaired vascular response following vaccination, it appeared that, as well as partially reversing endothelial dysfunction once established, *pre-treatment* with ascorbic acid *prevented* the development of endothelial dysfunction. Ascorbic acid could prevent NO breakdown by stopping the reaction of NO with oxidant radicals or prevent the oxidation and inactivation of the NOS co-factor tetrahydrobiopterin([378]). $BH_4$ is inactivated to $BH_2$, which is ineffective as a NOS co-factor and in this state NOS enzyme itself synthesises oxygen radicals in preference to NO. As a result of these observations a series of experiments were

performed to test the following hypotheses: (i) that systemic pre-treatment with ascorbic acid could prevent the development of inflammatory endothelial dysfunction, (ii) that tetrahydrobiopterin supplementation after vaccination could restore endothelial function and (iii) that the effect of tetrahydrobiopterin was altered by pre-treatment with ascorbic acid.

## 5.2 Protocols

### 5.2.1 Subjects

Twelve male and female subjects aged 22 to 40 were studied. No subjects had received typhoid vaccination in the preceding 6 months. Individuals were studied at the same time of day on two consecutive afternoons.

| | **Ascorbic Acid Group** | **Control Group** | Comparison P value |
|---|---|---|---|
| **Number of Subjects** | 6 | 6 | |
| **Age** (years) | 24 (2) | 26 (7) | 0.4 |
| **Sex** (male/female) | 5/1 | 5/1 | |
| **Systolic Blood Pressure** (mmHg) | 129 (12) | 123 (5) | 0.3 |
| **Diastolic Blood Pressure** (mmHg) | 74 (9) | 74 (7) | 0.9 |
| **Medications** | Nil | Nil | |

**Table 5.1 Baseline characteristics of subjects in pre-treatment study (expressed as mean (SD)) with comparison between groups showing no significant differences**

### 5.2.2 Generation of an inflammatory response

Salmonella typhi capsular polysaccharide vaccine 0.025mg (Typhim Vi, Pasteur Merieux MSD) was injected into the gluteus muscle at 8am on the morning of the second day of the study (section 2.3).

### 5.2.3 Assessment of forearm blood flow

Venous occlusion plethysmography was used to measure forearm blood flow 16 hours before vaccination (control) and 8 hours following vaccination (detailed in section 2.2.1).

Forearm blood flow in response to intra-arterial infusion of the vasodilators bradykinin (BK; 20, 40, 80pmol/min; each dose for 3 minutes) and glyceryl trinitrate (GTN; 8, 16 and 32 nmol/min; each dose for 3 minutes) were assessed 16 hours before and 8 hours after vaccination. The order of the infusions was randomised. Saline was infused for 15 minutes between drug infusions to allow restoration of baseline flow.

On the first day, after the initial assessment of endothelial function, the intra-arterial needle was removed and a venous cannula inserted under local anaesthetic. Blood was taken for analysis and subjects were randomised, using a number table stratified by gender, to receive intravenously either ascorbic acid (1.5g, dissolved in 30ml of 0.9% saline) or 30ml of 0.9% saline alone over 20 minutes in a double blinded fashion.

On the second day, 8 hours after vaccination endothelial function was again assessed and then tetrahydrobiopterin ($BH_4$; 500mcg/min) was infused intra-arterially for 15 minutes and the dose responses to BK and GTN were repeated.

In five individuals the effect of $BH_4$ on basal blood flow was determined in the absence of an acute inflammatory stimulus (by Dr L Mayahi).

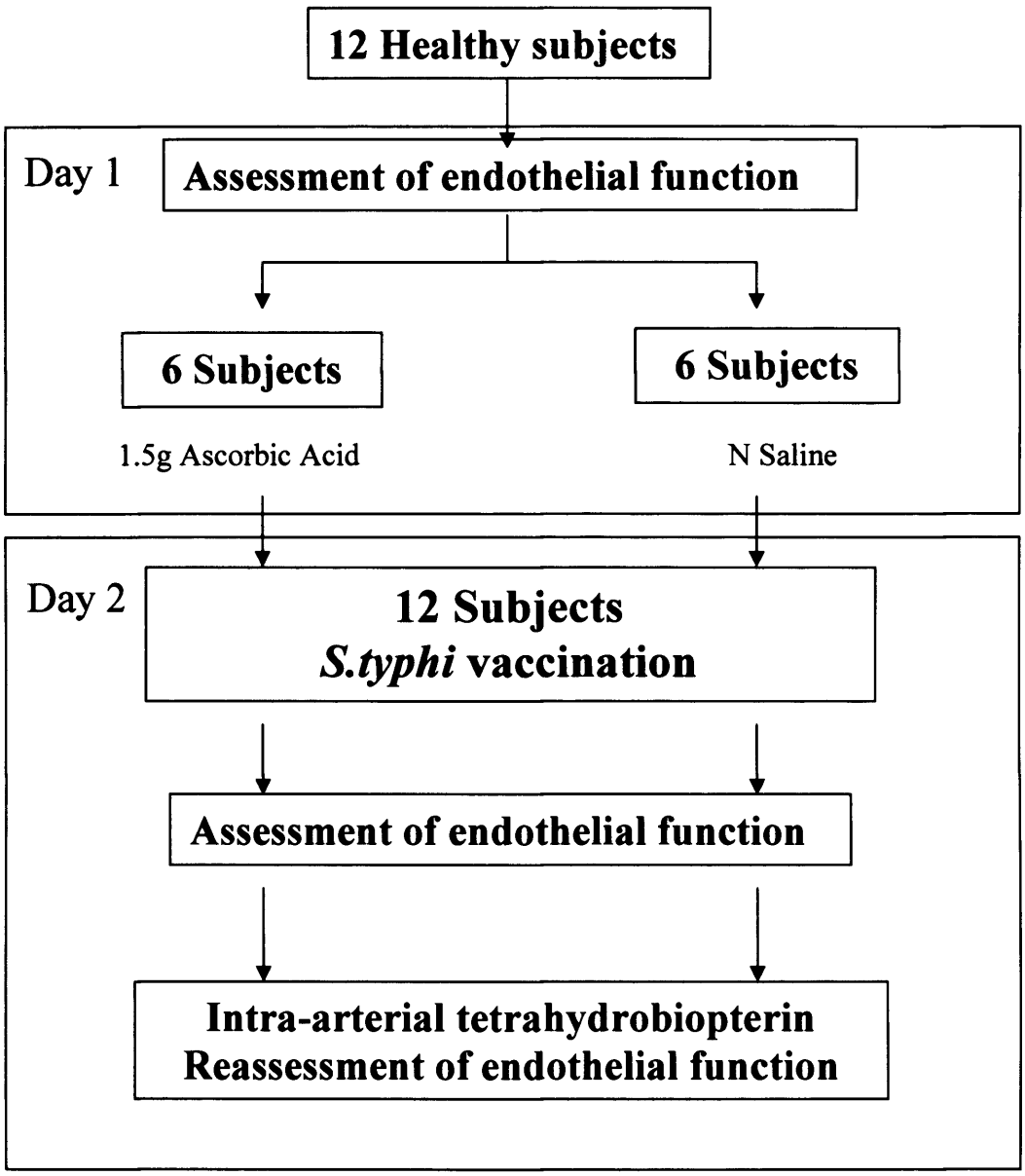



**Figure 5-1: Flow diagram of protocol for the ascorbic acid pre-treatment and tetrahydrobiopterin study**

### 5.2.4 Measurement of Cytokines, Serum Total Anti-Oxidant Status and Serum Ascorbic Acid

Blood samples were taken on the first day and then before and at 4 and 8 hours after vaccination for the measurement of cytokines, total anti-oxidant status (TAOS) and ascorbic acid. In all subjects the plasma was used to measure interleukin-6 (IL-6), interleukin-1 receptor antagonist (IL-1Ra), tumour necrosis factor α (TNFα) and TAOS. Ascorbic acid concentration was measured in samples pre-treated with metaphosphoric acid to stabilise the vitamin. Individual assays are described in section 2.6.

### 5.2.5 Calculations and statistical analysis

Analysis of forearm blood flow was performed as described in section 2.2.1. Results are expressed as mean ±SEM unless otherwise stated. Cumulative dose-response curves were constructed for all drugs and the area under the curve (AUC) calculated. Responses were compared by paired Student's t-test. The Wilcoxon sign ranked test was used for non-parametric data. Due to the change in basal flow following $BH_4$ infusion the post infusion data was compared by the repeated measures of covariance model (XTREG procedure in STATA8.2). Data was corrected for baseline flow in the control and active arms and the change in flow over the three doses of BK or GTN. The time course of the cytokine response was expressed as the AUC and analysed by a one-sample t test. Cytokine and ascorbic acid responses between groups were compared by 2-way ANOVA, while TAOS responses between groups were compared by unpaired Student's t-test. $P<0.05$ was considered statistically significant.

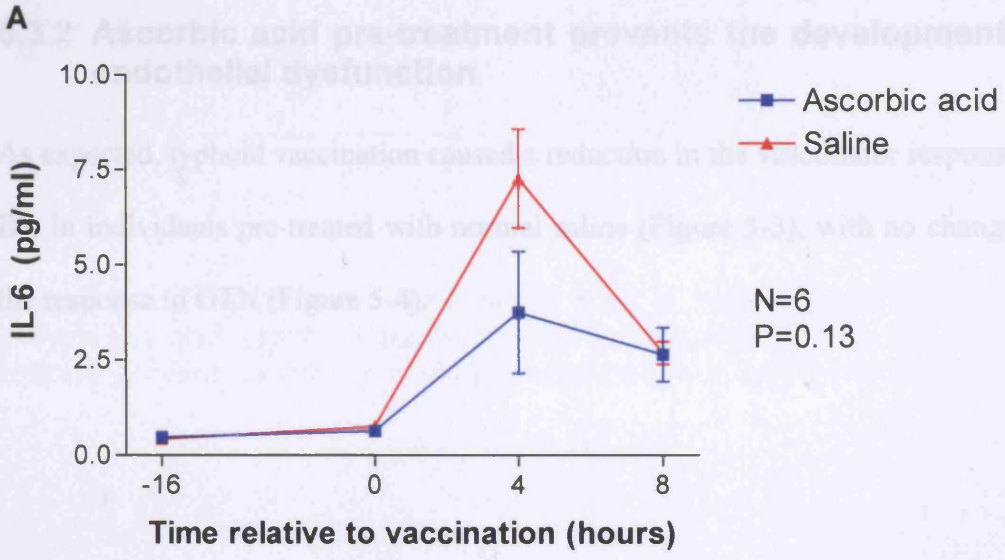
### 5.2.6 Drugs

BK was obtained from Clinalfa AG; GTN from Faulding Pharmaceuticals, $BH_4$ form Schricks Laboratories (Switzerland) and ascorbic acid from Medeva Pharma Ltd. All of the drugs were prepared as stock, solutions and stored at –20°C until use.

## 5.3 Results

### 5.3.1 Vaccination leads to an inflammatory response independent of ascorbic acid treatment

As with previous studies vaccination led to an increase in the concentration of circulating cytokines. There was a significant peak of IL-6 at 4 hours (0.69±0.2pg/ml at baseline vs. 5.5±3.9pg/ml at 4 hours; P=0.001, N=12). In contrast there was little change in the concentration of TNFα (6.3±2.3pg/ml at baseline vs. 6.3±2.3pg/ml at 8 hours; P=0.9, N=12). The cytokine response to vaccination was not affected by pre-treatment with ascorbic acid (Figure 5-2 A and B).



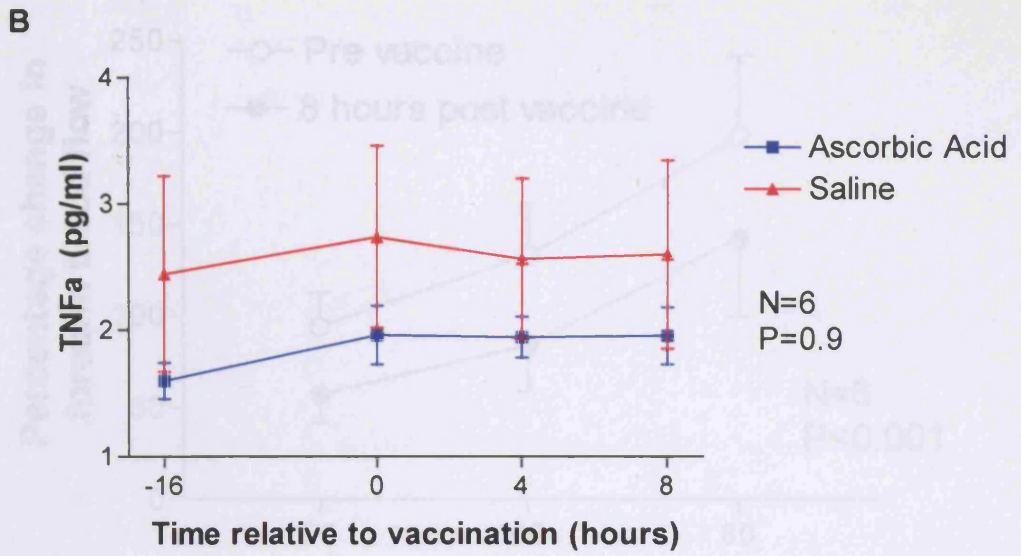



**Figure 5-2: Change in serum concentration of IL-6 (A) and TNFα (B) in the time following vaccination. Although there was a significant rise in IL-6 at 4 and 8 hours, ascorbic acid treatment did not modify the cytokine response (P=0.13 by 2-way ANOVA, P=0.17 by t test for 4 hour time point)**

There were no significant differences in the IL1-Ra responses between groups (data not shown).

### 5.3.2 Ascorbic acid pre-treatment prevents the development of endothelial dysfunction

As expected, typhoid vaccination caused a reduction in the vasodilator response to BK in individuals pre-treated with normal saline (Figure 5-3), with no change in the response to GTN (Figure 5-4).

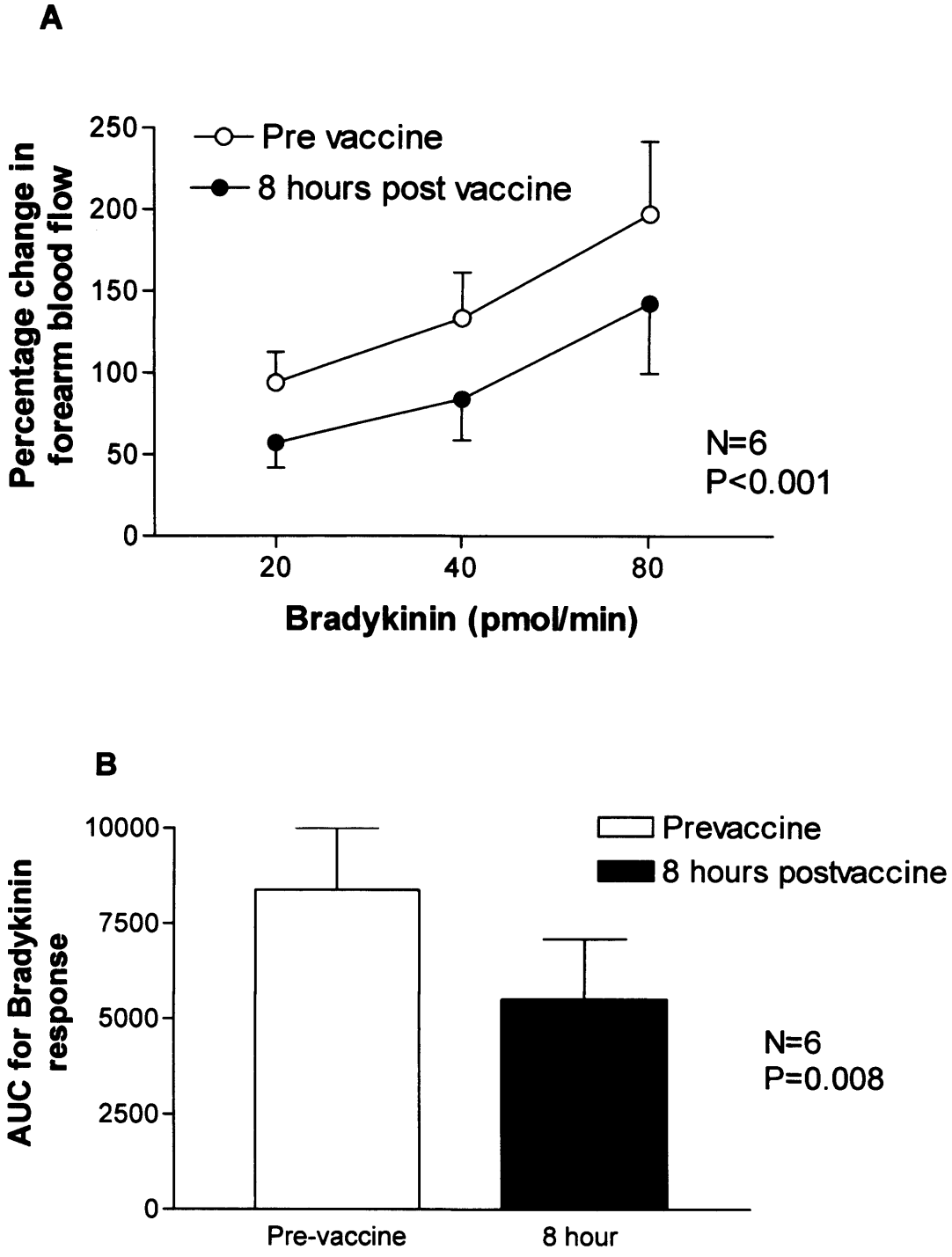



**Figure 5-3: Change in forearm blood flow in response to bradykinin before (○) and eight hours following vaccination (●) in the saline-treated control group (A), and expressed as AUC (B) (n=6, P<0.001)**

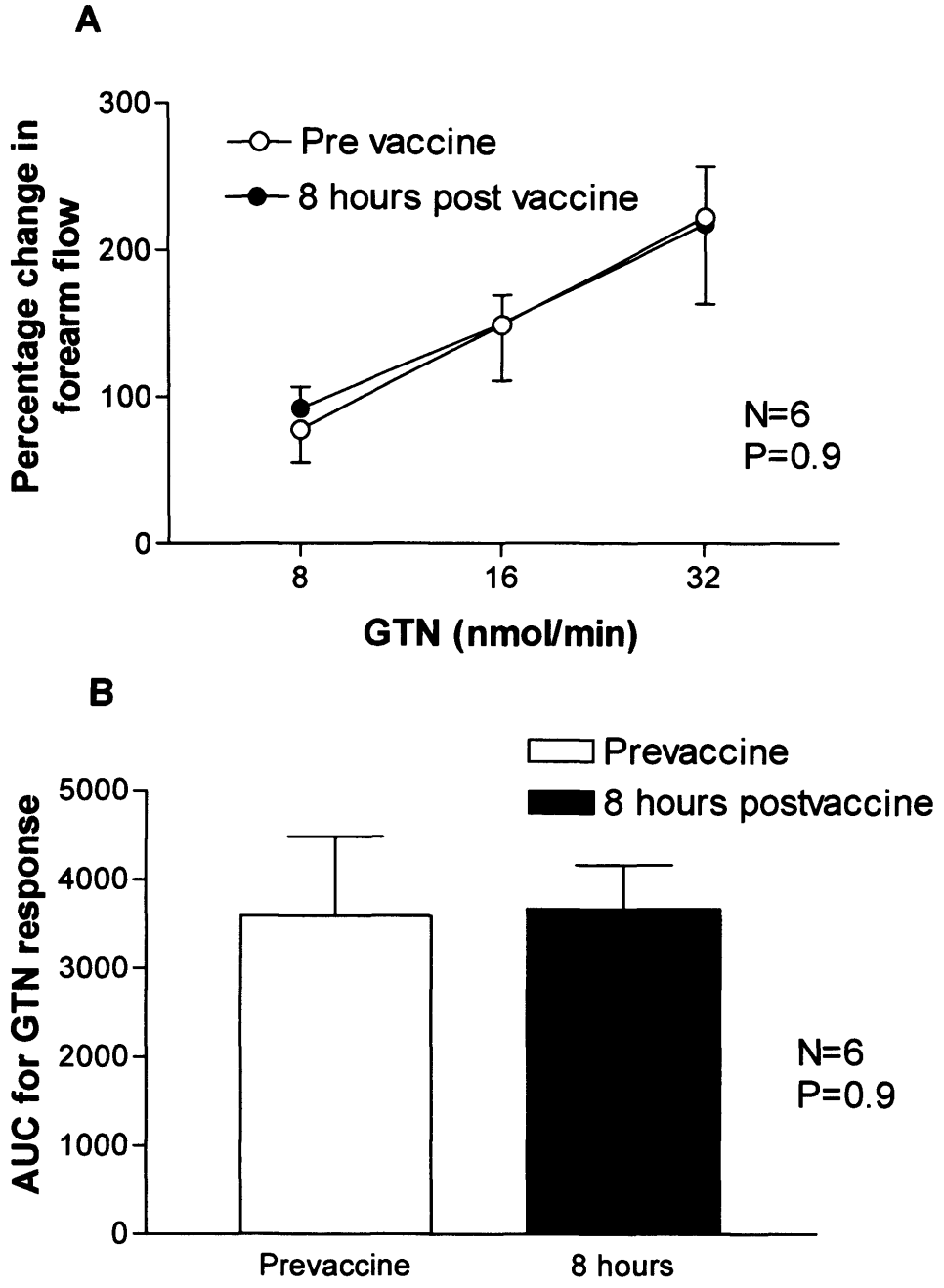



**Figure 5-4: Change in forearm blood flow in response to GTN before (○) and eight hours following vaccination (●) in the saline-treated control group (A), and expressed as AUC (B) (n=6, P=0.9)**

In contrast, individuals who were pre-treated with ascorbic acid (1.5g IV) showed an augmentation rather than attenuation in vasodilator response to BK (Figure 5-5), with no change in response to GTN (Figure 5-6).

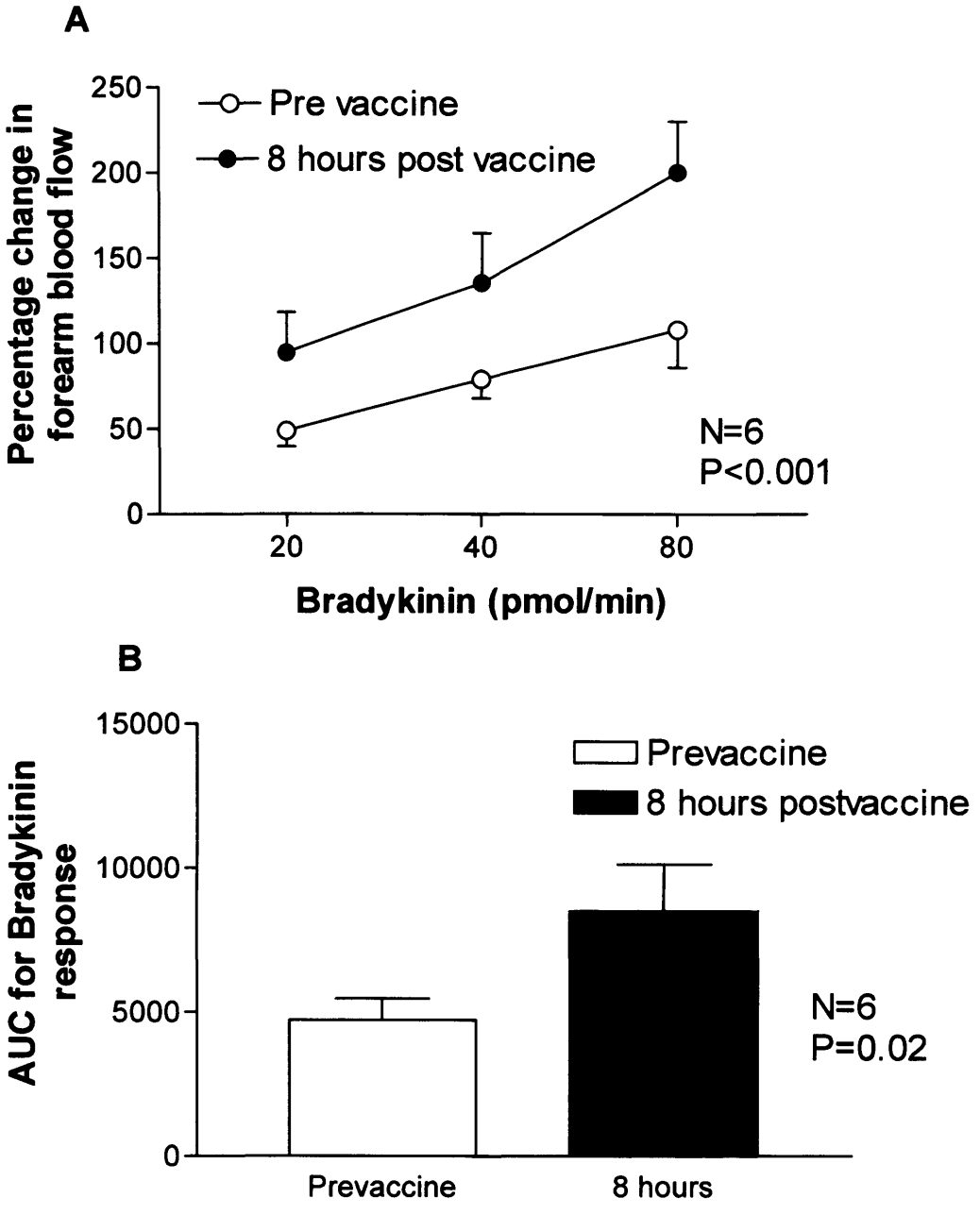



**Figure 5-5: Change in forearm blood flow in response to bradykinin before (○) and eight hours following vaccination (●) in the group pre-treated with ascorbic acid (A), and expressed as AUC (B) (n=6, P<0.001)**

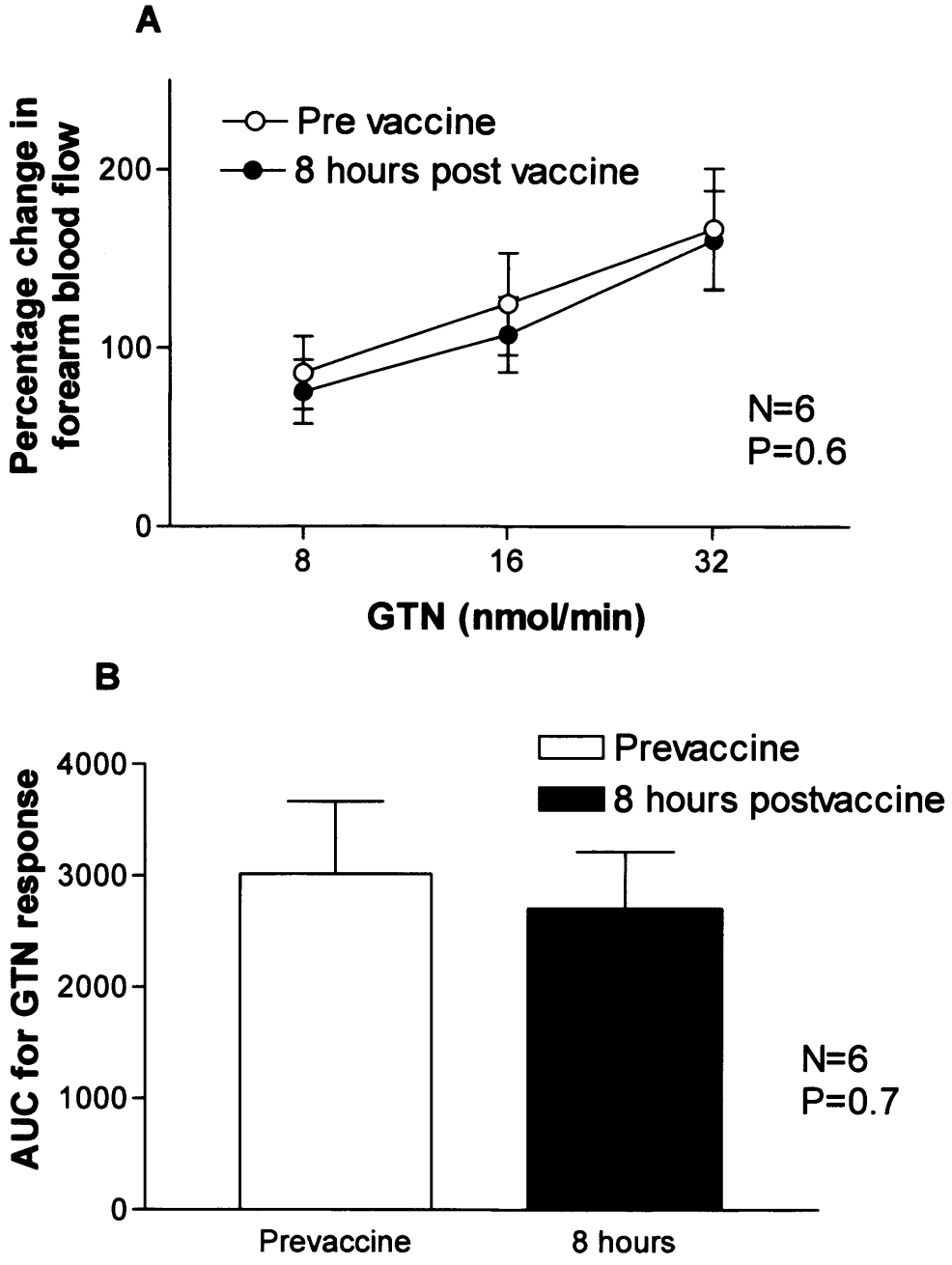



**Figure 5-6: Change in forearm blood flow in response to GTN before (○) and eight hours following vaccination (●) in the group pre-treated with ascorbic acid (A), and expressed as AUC (B) (n=6, P=0.6)**

### 5.3.3 Vaccination alters the basal response to tetrahydrobiopterin

Following vaccination the control subjects showed an increase in forearm blood flow following a 15-minute infusion of tetrahydrobiopterin (Figure 5-7; N=6,

P=0.02). There was no significant change in flow in individuals pretreated with ascorbic acid (Figure 5-8) or those, in a separate set of experiments, infused with BH4 in the absence of vaccination (Figure 5-9).

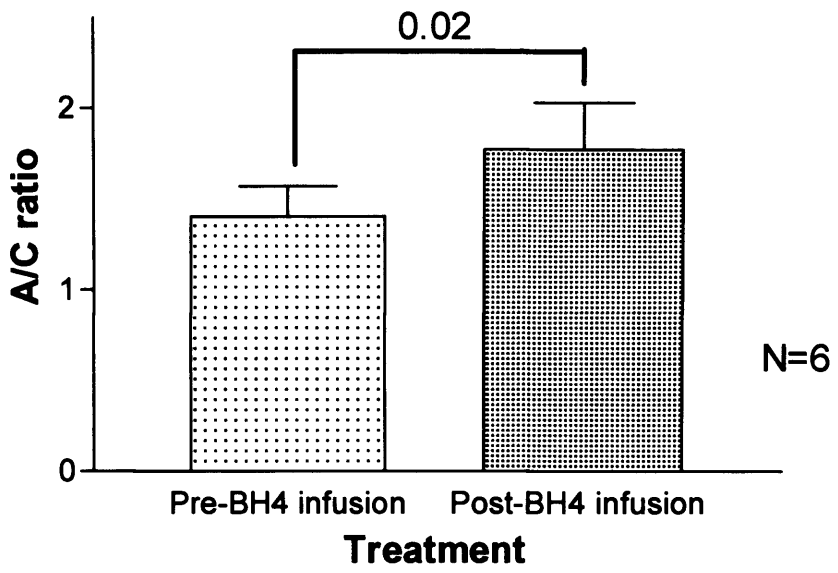



**Figure 5-7: Increase in relative forearm flow (expressed as active [A] over control [C] arm) after infusion of $BH_4$ (500mcg/min for 15 minutes) in control group following vaccination (N=6, P=0.02)**

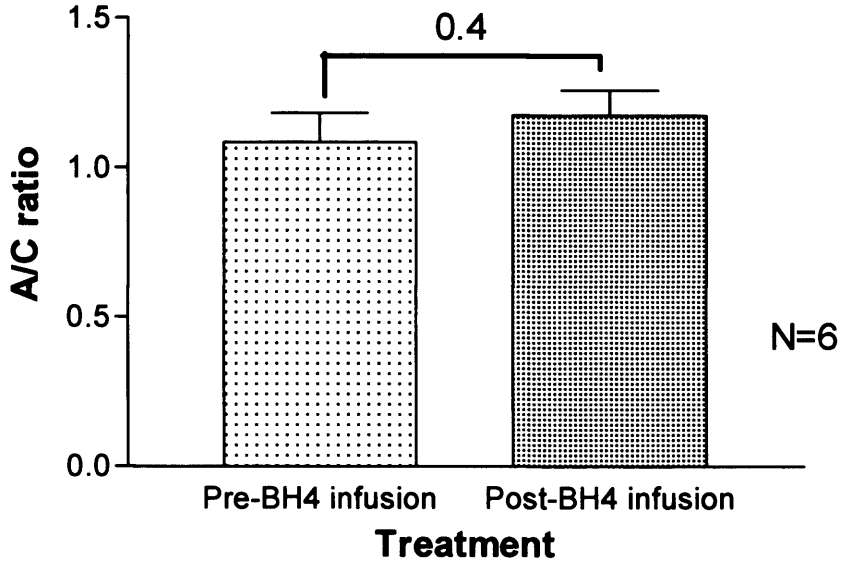



**Figure 5-8: Absence of change in relative forearm flow (expressed as active [A] over control [C] arm) following infusion of $BH_4$ (500mcg/min for 15 minutes) following vaccination in group pre-treated with ascorbic acid (N=6, P=0.4)**

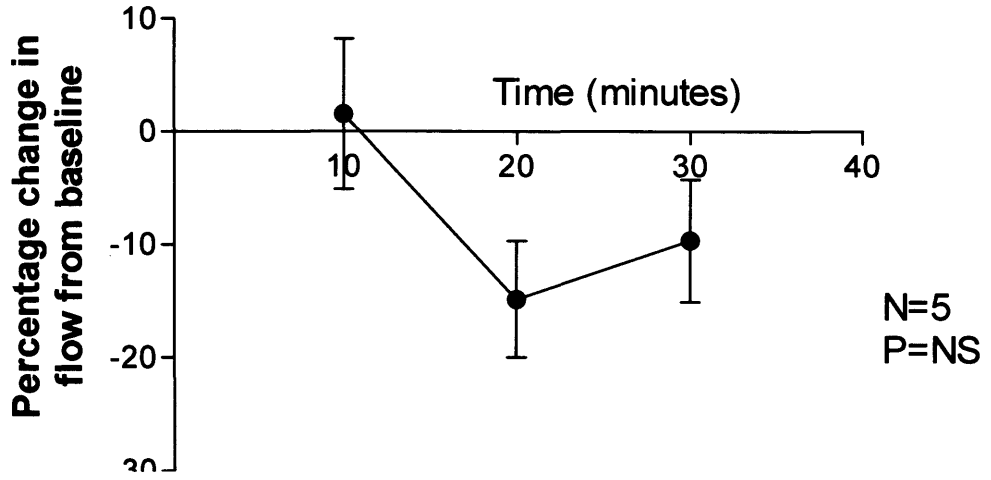



**Figure 5-9: Absence of change in basal flow (expressed as percentage change from baseline) in individuals infused with BH4 in the absence of the vaccine inflammatory stimulus at 10, 20 and 30 minutes.**

The change in basal flow make interpretation of subsequent dose-response curves difficult as all the following assessments depend on the basal flow([449]). To try and account for this a repeated measures of covariance model was applied and showed that following $BH_4$ infusion there was an increased response to BK in only the group not pretreated with ascorbic acid (table 5.2). In contrast the GTN response was not affected by BH4, unless the vessel had been pretreated with ascorbic acid (table 5.2)

| | Change in BK response after BH4 in untreated group | Change in GTN response after BH4 in untreated group | Change in BK response after BH4 in pretreated group | Change in GTN response after BH4 in pretreated group |
|---|---|---|---|---|
| β coefficient | 0.18 | 0.091 | 0.058 | 0.15 |
| 95 % CI | 0.007 to 0.3 | -0.2 to 0.2 | -0.5 to 0.16 | 0.08 to 0.22 |
| P value | 0.002 | 0.1 | 0.3 | <0.001 |

**Table 5.2 Result of regression analysis indicating that BK vasodilates vessels more following $BH_4$ infusion, though only in the group not pretreated with ascorbic acid. The reverse is true for GTN response.**

### 5.3.4 Changes in vascular responses due to ascorbic acid treatment are not mediated by alterations in oxidant stress

In all subjects TAOS decreased 8 hours following vaccination (Figure 5-10; N=12, P=0.03), though there was no significant difference in the reduction in TAOS between the ascorbic acid treated groups and controls (Figure 5-11; N=6, P=0.35).

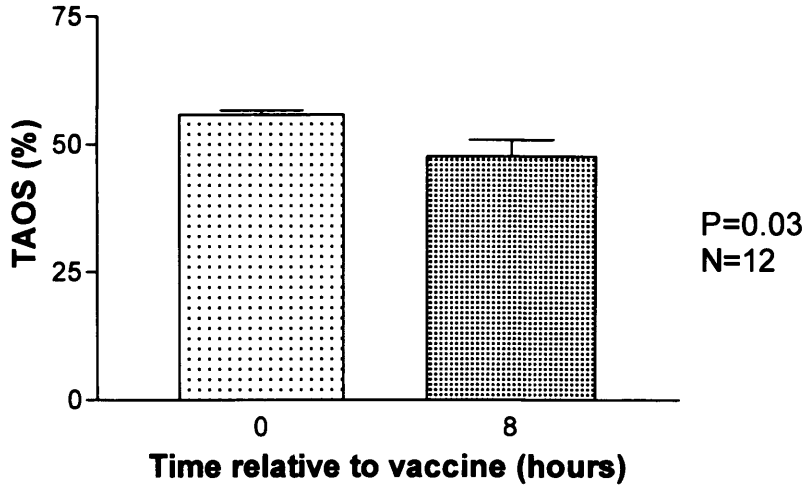



**Figure 5-10: Vaccination leads to a significant fall in total anti-oxidant status at 8 hours after stimulus compared to before (N=12, P=0.03)**

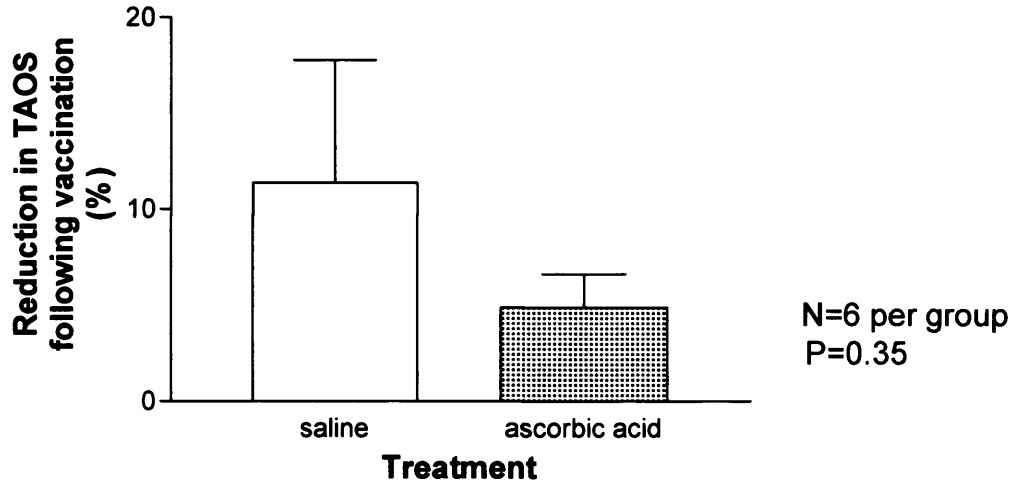



**Figure 5-11: The fall in total anti-oxidant status is not different between individuals treated with ascorbic acid and controls (N=6, P=0.35)**

In the subjects pre-treated with ascorbic acid their serum concentration increased significantly from baseline (-16 hours) to the time of vaccination (0 hours) (N=6, P=0.01), and then returned to normal by the time of repeat vascular study, 8 hours later (N=6, P=0.14). Repeated measures ANOVA was significant (P<0.001 overall) and by Bonferroni post-hoc analysis there was a significant increase in ascorbic acid concentration at the time of vaccination relative to baseline

(P<0.05). In the control group ascorbic acid concentrations did not change at any point (N=6, P=0.23 from baseline to vaccination and P=0.9 from baseline to 8 hours after vaccination) (Figure 5-12). In the control group the repeated measures ANOVA was not significant (P=0.3).

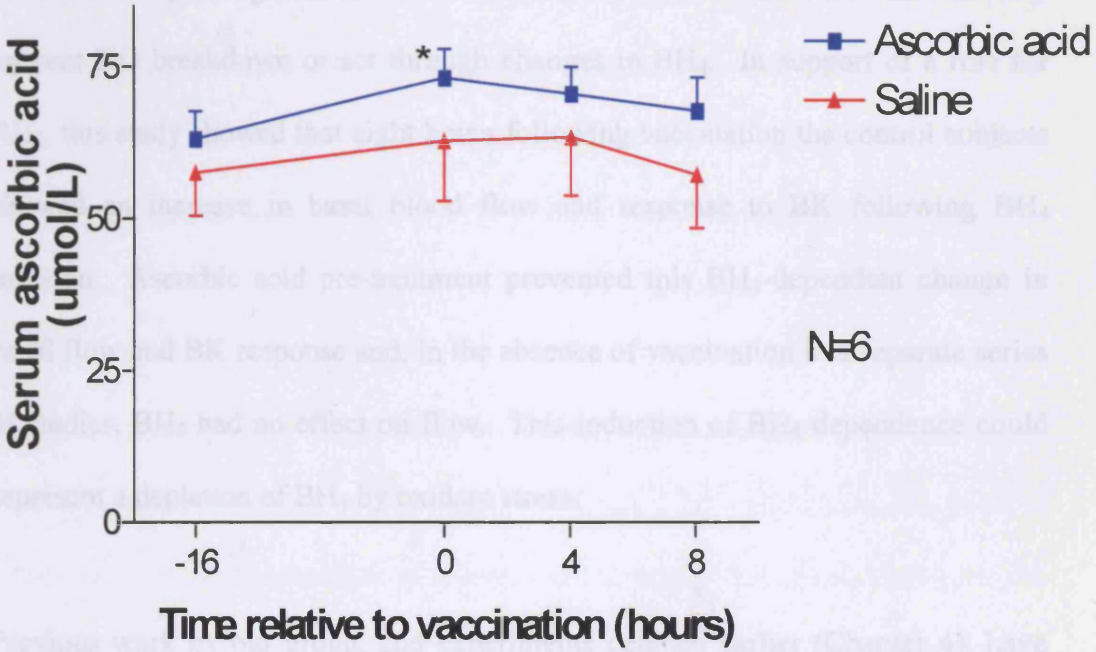



**Figure 5-12: Serum concentration of ascorbic acid from before infusion of ascorbic acid (-16 hours), prior to vaccination (0 hours) and 4 and 8 hours later (point of vascular study). There is a significant rise in concentration from baseline (-16 hours) to vaccination (0 hours) only in the ascorbic acid treated group (P=0.01, vs P=0.23 for control; * on graph) and even in that group this normalises by 8 hours (P=0.14) (N=6 in each group).**

## 5.4 Discussion

In this chapter it has been shown that vaccination-induced endothelial dysfunction can be completely prevented by the pre-administration of ascorbic acid. This led

to a significantly increased concentration of ascorbic acid at the time of the vaccination, which had normalised by the time endothelial function was reassessed. There was an equivalent fall in total anti-oxidant status of the serum, at the time of the repeated study of the endothelial function, irrespective of pre-treatment. The mechanism by which the ascorbic acid acts to prevent inflammation-induced endothelial dysfunction in this context is not clear. It may be mediated by changes in intracellular anti-oxidant stores that then either directly prevent NO breakdown or act through changes in $BH_4$. In support of a role for $BH_4$, this study showed that eight hours following vaccination the control subjects showed an increase in basal blood flow and response to BK following $BH_4$ infusion. Ascorbic acid pre-treatment prevented this $BH_4$-dependent change in basal flow and BK response and, in the absence of vaccination in a separate series of studies, $BH_4$ had no effect on flow. This induction of $BH_4$ dependence could represent a depletion of $BH_4$ by oxidant stress.

Previous work by our group, and experiments detailed earlier (Chapter 4), have shown that vaccination leads to a reduction in the vasodilator response to bradykinin, indicating the development of endothelial dysfunction([50;324]). In the last chapter this reduction in bradykinin response was shown to be partially reversible by the infusion of ascorbic acid 8 hours after the stimulus. This is in keeping with the observation that there is an increase in oxidant stress within the serum at 8 hours following vaccination (Figure 4-3). Ascorbic acid may produce its effects by directly quenching reactive oxidant species([425]) or by stabilising the nitric oxide synthase cofactor $BH_4$([379]). In this chapter the pre-treatment with ascorbic acid completely prevented the development of endothelial dysfunction.

These studies show that the protective effect of ascorbic acid is not mediated by a reduction in the measured cytokine response, as occurred in the studies with anti-inflammatory dose aspirin pre-treatment([50]). It was possible that there was an alteration in cytokine responses that were not directly measured, or alternatively inadequate numbers were studied leading to a type II error. This is in contrast to a clinical study of inflammation in acute pancreatitis, in which high dose ascorbic acid led to a reduction in all inflammatory cytokines (including IL-6)([485]). The difference may be a dose and duration effect, as the clinical pancreatitis study employed 10g/day for a sustained period as opposed to a single 1.5g dose in this work.

Previous chapters have shown that vaccination leads to a detectable fall in the total anti-oxidant capacity of the blood at 8 hours after vaccination, the point at which endothelial dysfunction is reassessed. One potential explanation for the effects of pre-treatment with ascorbic acid was that it produces a prolonged increased concentration of this anti-oxidant in the serum and that this leads to a prevention of the fall in TAOS. This was not borne out in this work, as there was neither a sustained increase in ascorbic acid concentration nor a difference in the development of "oxidant stress" in the ascorbate treatment group when measured at 8 hours. It must be noted that at the time of vaccination the ascorbic acid concentration in pre-treated subjects was higher than that of controls and it is possible that this alters the response to the inflammatory stimulus, perhaps by intra-cellular changes, such as supplementing intra-cellular anti-oxidant stores that

could locally prevent degradation of $BH_4$, that cannot be measured by the techniques employed in this work.

During these experiments the basal peripheral vascular tone, as shown by basal flow prior to the dose-response curves, appears to become $BH_4$ dependent following vaccination. This is shown by the increase in flow following the infusion of $BH_4$ alone. By contrast, in the control non-vaccinated subjects and published studies([100]), $BH_4$ has no effect on the basal tone of "healthy" vessels. In individuals with traditional cardiac risk factors, such as diabetes([384]), smoking([380]) and hypercholesterolaemia([383]), and established coronary heart disease([382]), infusion of $BH_4$ improves the response to endothelial-dependent vasodilators, though it did not alter basal flow. These results suggest that an acute inflammatory stimulus acts to deplete or deactivate $BH_4$, or render the endothelium resistant to $BH_4$ making it possible for supplementation now to alter basal tone. Although not directly tested in these experiments supplementation is thought to act by altering the balance of NO to superoxide production by NOS in favour of the vasodilator. It cannot be discounted that $BH_4$ may be acting directly as an anti-oxidant and reducing NO breakdown, perhaps as well as augmenting its production.

Pre-infusion with ascorbic acid avoids the development of $BH_4$ dependence by the forearm vasculature. As such there is no change in the basal forearm flow after infusion of $BH_4$. It has been shown that ascorbic acid can increase the efficiency of NOS by the stabilisation of the essential co-factor $BH_4$([378;478]). This may be particularly relevant in this situation as it is known that when oxidised $BH_4$ loses

its ability to dimerise NOS it actually tends to favour the production of further oxygen free-radicals([363;371;379]). It is therefore possible that pre-treatment with ascorbic acid has its beneficial effects through an alteration in $BH_4$ bioavailability that is sustained to up to 8 hours following vaccination. Whether this occurs at the time of ascorbic acid infusion or later is not clear and exactly how it acts is also not apparent.

In a similar way there appeared to be an increase in the response to BK following $BH_4$, though only in the group not pretreated with ascorbic acid. This supports the concept of developing $BH_4$ dependence. The failure of $BH_4$ to increase flow following ascorbic acid pre-treatment could be due to maximal flow in these vessels prior to the infusion of the pterin, or that ascorbic acid prevented $BH_4$-dependence developing. Although regression analysis has been applied previously to situations where basal flow has altered([100]) this must be treated with caution. It is possible that changes in basal tone may make any subsequent responses to stimulants uninterpretable([449]). It is not clear why ascorbic acid pre-treatment increased the response to GTN following $BH_4$ infusion and this needs further investigation.

In animals and humans, bradykinin vasodilates in part by an NO-dependent mechanism, with a probable contribution from endothelium-derived hyperpolarizing factor([57;472]). It is therefore possible for the ascorbic acid pre-treatment to be acting by improving availability of NO or EDHF post-vaccination. As indicated above this does not seem to be by a direct effect on oxidant stress at the time of the plethysmography study. An EDHF dependent effect is not

explored in these studies. The data on the changes in effect of $BH_4$ suggest that at least in part the ascorbic acid acts on the NO pathway. Ascorbic acid in these experiments was given systemically by intravenous injection 16 hours before the stimulus. Further experiments would be needed to determine the duration of effect of this dose and the outcome if the anti-oxidant was given either orally or locally. Similarly the effect of other anti-oxidants, such as vitamin E, or a combination of agents is not clear.

In conclusion the experiments in this chapter suggest that inflammation may in part act through alteration in $BH_4$ bioavailability and that this can be prevented by pre-treatment with ascorbic acid. Further experiments are needed to determine the applicability of these observations to other inflammatory stimuli, the duration of the effect and their importance to the development of unstable vascular disease.

# 6 Determination of the direct vascular effects of C-reactive protein *in vitro*

## 6.1 Background

The introduction to this thesis describes the effects of infection and inflammation on vascular function. Acute sepsis may be accompanied by profound vasodilatation and hypotension([486]) and, following the resolution of acute infection, there is a transient increase in the risk of atherothrombotic events([149-151]). Chronic, sub-clinical infection or inflammation may also contribute to atherogenesis([22]), since a variety of inflammatory markers([152;213]), persistent infection([161;179]), and chronic autoimmune disorders([182]) are all associated with an increase in the rate of clinical cardiovascular disease. The mechanisms that underlie these observations are incompletely understood, but it is possible that products of the inflammatory response contribute both to the vasodilatation of sepsis, and modulate the development and stability of atheromatous plaques, by altering the release of vasoactive mediators such as nitric oxide (NO) by the vascular endothelium([23;104;109]).

It has been known for many years that the concentration of CRP, a member of the pentraxin family, in plasma increases during acute infective or inflammatory episodes, as part of the innate immune response. More recently, it has also been shown that the baseline level of CRP is predictive of future cardiovascular events. Whilst the role of CRP in host defence, and as a clinically useful marker of infectious disease and inflammation is well established, the possible biological effects of CRP on the cardiovascular system are less clear.

The *in vivo* model used in the previous chapters suggests that products of the inflammatory response could act through oxidant stress and the availability of $BH_4$ to produce endothelial dysfunction. Given the strong link between CRP and vascular events in epidemiological studies, and the published evidence for a proatherogenic role for CRP, the direct effects of CRP on vascular function were investigated in the experiments described in this chapter.

As no specific inhibitor of CRP for use *in vivo* as yet exists these studies were carried out *ex vivo*. Concerns exist as to the purity of commercially available CRP, and specifically the presence of preservatives that may directly influence cellular function. As a result these studies were performed with highly purified CRP that was not stored with any preservatives.

## 6.2 Protocols

### 6.2.1 Isolation and purification of human CRP

Human CRP was purified from human ascites by Dr M Pepys' group at the Royal Free Hospital as previously described in section 2.6.1.2([457]). SDS gel electrophoresis demonstrated a single band corresponding to an approximate size of 23K. CRP and its vehicle contained <0.5ng/ml bacterial lipopolysaccharide (LPS) by Limulus chromogenic assay (BioWhittaker Europe).

### 6.2.2 Commercially available human CRP

CRP is available commercially from Calbiochem (Beeston, Nottingham, UK) and is shipped at a concentration of 1mg/mL in solution containing 140mM NaCl, 20mM Tris-HCl, 2mM $CaCl_2$ and 0.05% sodium azide (pH 7.5).

In experiments where commercial CRP was used it was first dialysed three times against a large volume of buffered solution of the same contents as detailed above with the exception that it did not contain sodium azide. Final concentrations were confirmed to not be significantly changed. These procedures were performed by Dr G Hirschfield at the Royal Free Hospital London.

### 6.2.3 Organ bath studies

Endothelium-intact or denuded rings of thoracic aorta from male Sprague-Dawley rats or human internal mammary artery were incubated in 0.5mL serum-free Dulbecco's modified Eagle's medium containing human CRP (2-200mg/L) or buffer in 95% $O_2$/5% $CO_2$ at 37°C for 4 hours. Following incubation, tissues were mounted in an organ bath containing Krebs solution as described in section 2.1.2.

Tissues were maximally contracted with KCl ($4.8 \times 10^{-2}$M) and then washed for 30 minutes before endothelial integrity was assessed by contraction with phenylephrine (PE; $10^{-7}$M) and subsequent relaxation with acetylcholine (ACh; $10^{-6}$M) as described in section 2.1.2.2. Rings were considered to have intact endothelium if ACh caused a greater than 50% reversal of PE contraction. Rings in which the endothelium was removed by gentle rubbing exhibited < 1% relaxation to ACh.

Following incubation with CRP or vehicle, concentration-response curves to PE ($10^{-9}$ – $10^{-5}$M) were constructed, and rings were then submaximally (80%) pre-contracted with PE before construction of concentration-response curves to ACh or sodium nitroprusside (SNP) ($10^{-9}$ – $10^{-5}$M).

Following incubation with CRP or vehicle, vessels were pre-incubated with nitro-L-arginine methyl ester (L-NAME; $3x10^{-4}$M for 30 minutes) or 1400W (N-(3-(Aminomethyl)benzyl)acetamidine; $10^{-5}$M for 30 minutes) to block NO synthesis, actinomycin D ($10^{-5}$M, for 4 hours) to block protein synthesis, methoxyacetylserotonin ($10^{-4}$M for 30 minutes) to block tetrahydrobiopterin synthesis, polymixin (10μg/ml) to bind LPS, or RMM (8.69mM) a novel low molecular weight inhibitor of CRP binding (Pepys MB et al. personal communication). In a further set of experiments the relaxant effect of D- or L-arginine ($3x10^{-4}$M) on vessels contracted submaximally with PE was ascertained. In all experiments the tissue rings were weighed at the end of the protocol and shown to be the same across all groups.

### 6.2.4 Protein expression studies

Aortic rings were incubated for 4 hours with buffer, CRP (200mg/L) or LPS (*Salmonella typhosa*; 500ng/ml) at 37°C and then protein expression of eNOS, iNOS and GTPCH-1 was determined as described in section 2.5.1.

### 6.2.5 Endothelial cell culture

Passage five human coronary aortic endothelial cells (HCAECs; Promocell, Heidelberg, Germany) were grown to 80% confluence and then treated as described in section 2.4.1.1 with various formulations of CRP or vehicle. Cells were then harvested and protein expression determined (see section 2.5.1). In separate experiments cell viability following incubation was determined with the thiazolyl blue tetrazolium blue (MTT) assay (see section 2.4.1.3).

### 6.2.6 Protein expression by Western blotting

Protein expression was determined with the following primary antibodies: rabbit anti-human eNOS (c-20) and rabbit anti-mouse iNOS (m-19)(Santa Cruz Biotechnology, California USA) and GTPCH-1 anti-peptide antibody (raised against amino acids 17-45 from human sequence). According to the methods described in section 2.5.1. Coomassie staining of gels and membranes was used to confirm equal protein transfer.

### 6.2.7 Determination of NO production and pH in a mouse endothelial cell line

As described in the methods section 2.4.2, experiments were performed using a mouse cell line that over-expressed GTP cyclohydrolase-1 feedback regulatory protein (GFRP). The rationale was to determine whether transgenic inhibition of GTPCH-1 activity would abolish the effect of CRP. Two experiments were conducted in this model. Firstly NO production was measured with a Griess assay, and secondly, the effect of different CRP preparations upon the pH of the

culture medium was investigated. The first aim of these experiments was to investigate whether CRP increased NO production, and to test whether this was mediated through GTPCH-1 expression. The second was to determine whether the pH of the culture media, during experimental conditions, was altered by the sodium azide component of commercial CRP preparations.

The two assays are described in detail in the methods section (2.6.6 and 2.6.7). The Griess assay was used in all cells, whereas the colorimetric assay of pH was only performed on the untransfected group. Treatment groups consisted of control, pure isolated human CRP (50mg/L), CRP sourced from Calbiochem (50mg/L), Calbiochem CRP that had been dialysed against buffer to remove all sodium azide (50mg/L) and a positive control of 10ng/ml TNFα, 100unit/ml interferon γ and 5μg/ml LPS.

In the pH experiments only a further positive control in the form of 1% and 0.1% myxothiazole (a respiratory chain inhibitor) was added. All incubations were performed for 24 hours.

### 6.2.8 Cohort study

Plasma samples from 579 healthy middle-aged male participants from the Northwick Park Heart Study II, a prospective cohort study of cardiovascular risk factors, were used to study correlations between plasma neopterin concentration (as an index of pterin pathway activation) and plasma CRP concentration. Details of the design, recruitment and follow-up have been described elsewhere([487]). C-reactive protein was measured on a BN Prospec (Dade Behring, Milton Keynes,

UK). Inter-assay and intra-assay coefficients of variation were <4% and <2% respectively with a detection limit of 0.20 mg/l. Plasma neopterin was measured by commercially available ELISA (Brahms, Hennigsdorf, Germany). The manufacturer's lower limit of detection of this assay was 2nmol/L, with an interassay CV of 9.6% at 7.19nmol/L and 5.14% at 65.42nmol/L. The statistician attached to the Northwick Park Heart Study, Jackie Cooper, performed the analysis.

## 6.3 Results

### 6.3.1 Purified, azide-free human CRP causes hyporeactivity in vascular rings through an endothelium-dependent mechanism

Human CRP (200mg/L) produced marked hyporeactivity to PE in rat aortic rings similar to that observed with Salmonella *typhosa* lipopolysaccharide (50ng/ml) (Figure 6-1: P<0.0001, 2-way ANOVA for CRP vs control). This effect was also seen in rings of human internal mammary artery (Figure 6-2; P<0.0001, 2-way ANOVA). Similar effects were seen at concentrations of CRP observed in health (2mg/L) or mild inflammation (20 mg/L) (Figure 6-3).

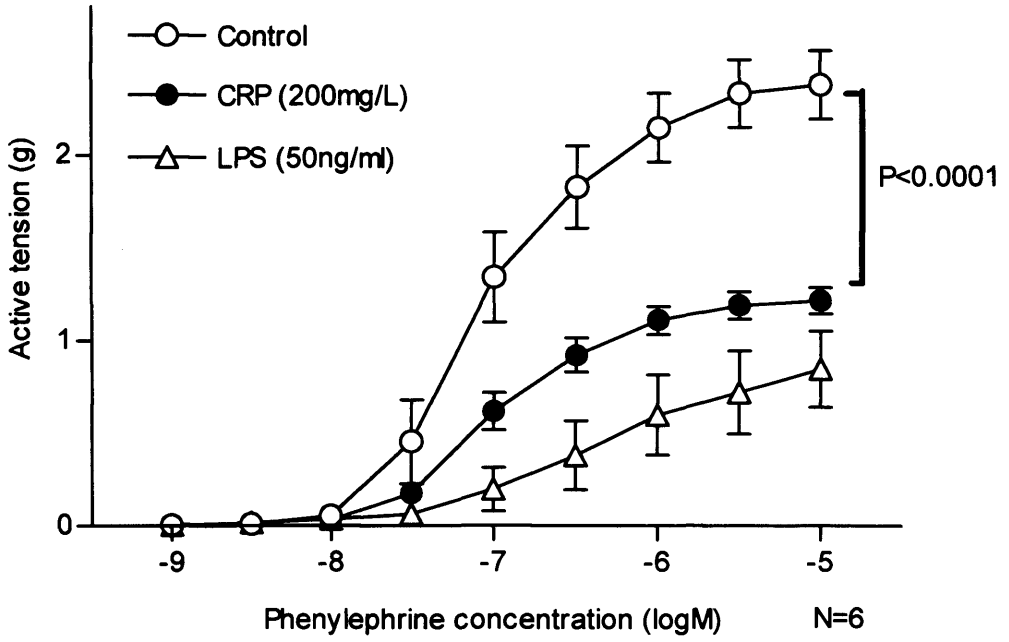



**Figure 6-1: Concentration response curve to PE in rat aortic rings; CRP (200mg/L for 4h; •) and LPS (50ng/ml for 4h; ▲) induce hyporeactivity compared to control (○) (N=6; P<0.0001 for CRP vs Control).**

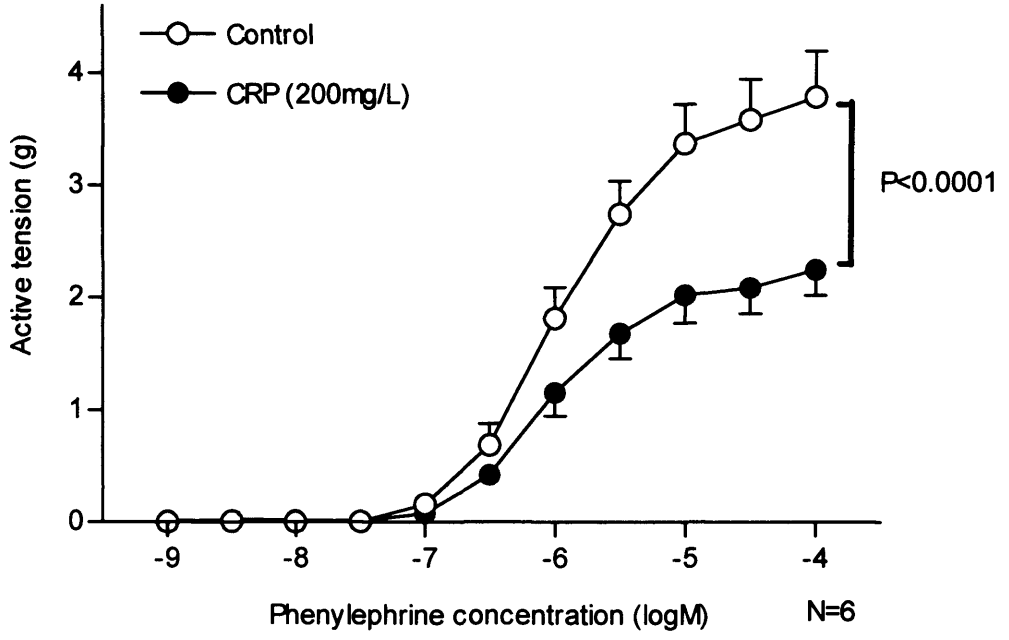



**Figure 6-2: Concentration response curve to PE in internal mammary artery rings; CRP (200mg/L for 4h; •) induced hyporeactivity compared to control (○) (N=6; P<0.0001 for CRP vs Control).**

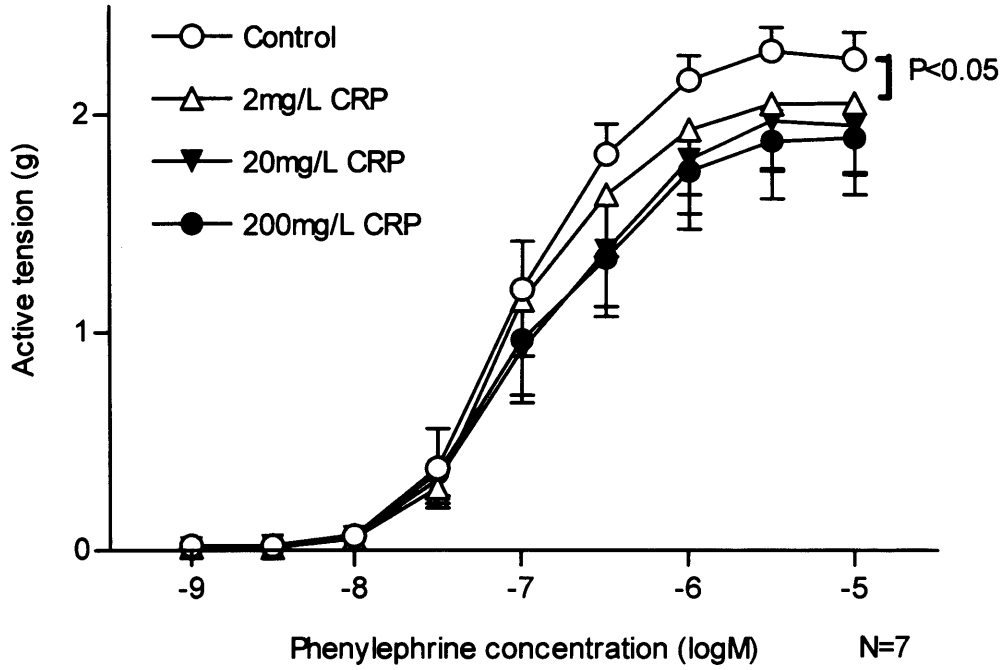



**Figure 6-3: Concentration response curve to PE in rat aortic rings; CRP at three concentrations (• 200mg/L, ▼20mg/L,Δ2mg/L) induced hyporeactivity compared to control (○)(N=7; P<0.05 for CRP 2mg/L vs Control).**

The effects were specific to the presence of CRP since they were abolished by preabsorption of CRP by Sepharose-phosphoethanolamine (Figure 6-4), and were blocked by RMM (a low molecular weight inhibitor of CRP binding) (Figure 6-5). The effects of CRP were not explained by the presence of LPS since the stock CRP solution and its solvent buffer contained <0.5ng/ml LPS, and pre-incubation with polymixin to bind LPS did not alter the effect of CRP (Figure 6-6).

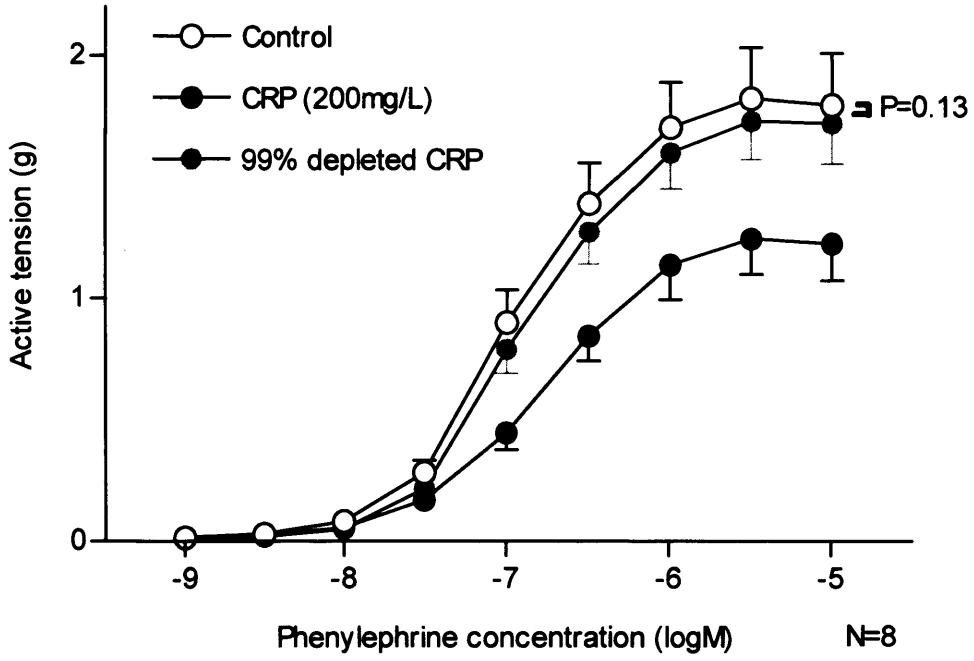



**Figure 6-4: Concentration response curve for rat aortic rings showing hyporeactivity to phenylephrine induced by CRP (•) relative to control (○) is abolished by pre-absorption of CRP with sepharose-phosphoethanolamine (•) (N=8; P=0.13 for depleted CRP vs Control).**

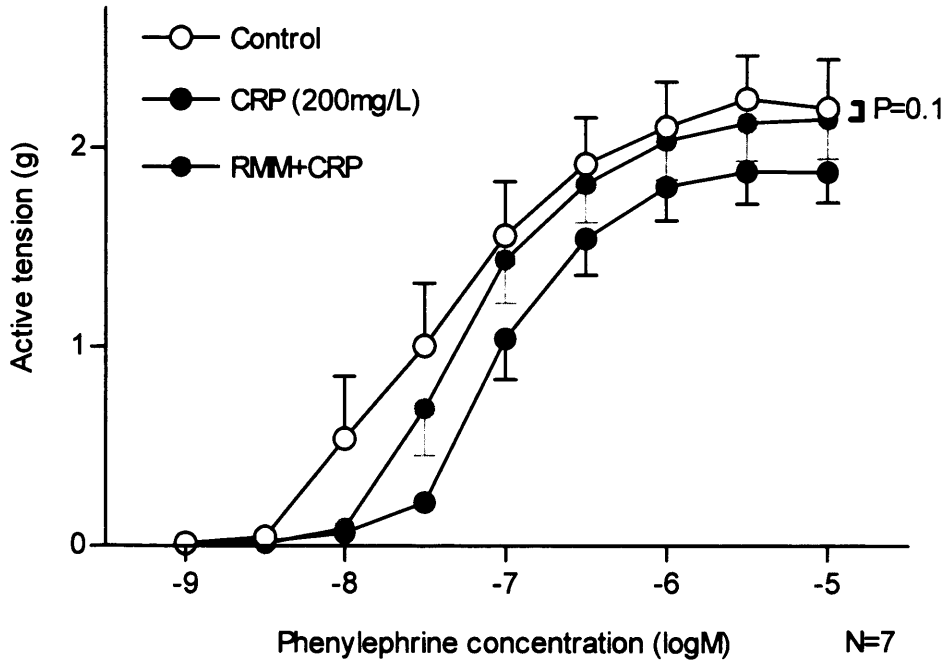



**Figure 6-5: Concentration response curve for rat aortic rings showing hyporeactivity to phenylephrine induced by CRP (•) relative to control (○) is abolished by treatment with specific inhibitor RMM(•) (N=7; P=0.1 for RMM and CRP vs Control).**

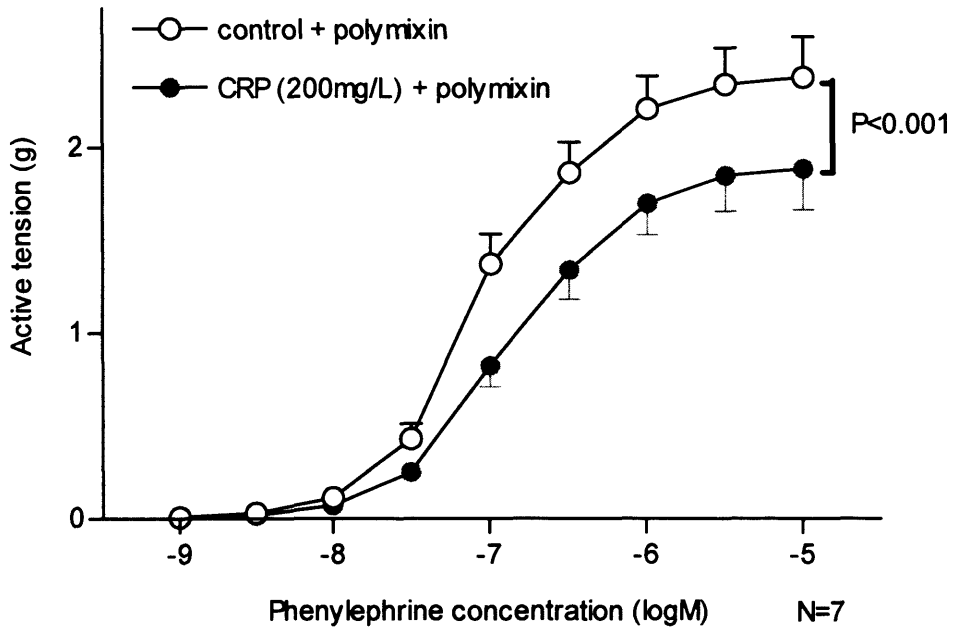



**Figure 6-6: Concentration response curve for rat aortic rings showing hyporeactivity to phenylephrine induced by CRP (•) relative to control (○) is not abolished by co-incubation with polymixin (10μg/ml) (N=7; P<0.0001 for CRP in the presence of polymixin vs polymixin only control). Contemporaneous positive and negative controls have been removed for clarity (P=0.02 for CRP vs control without polymixin).**

Endothelial denudation prior to CRP incubation (Figure 6-7), or non-isoform selective NOS inhibition with L-nitroarginine methyl ester (L-NAME) (Figure 6-8) after CRP incubation, prevented the effects of CRP, suggesting the PE hyporeactivity is mediated through physiological antagonism by endothelium-derived NO. Actinomycin D, which prevents protein synthesis, (Figure 6-9) blocked CRP-induced hyporeactivity to PE, indicating further that this action of CRP relies on new protein synthesis.

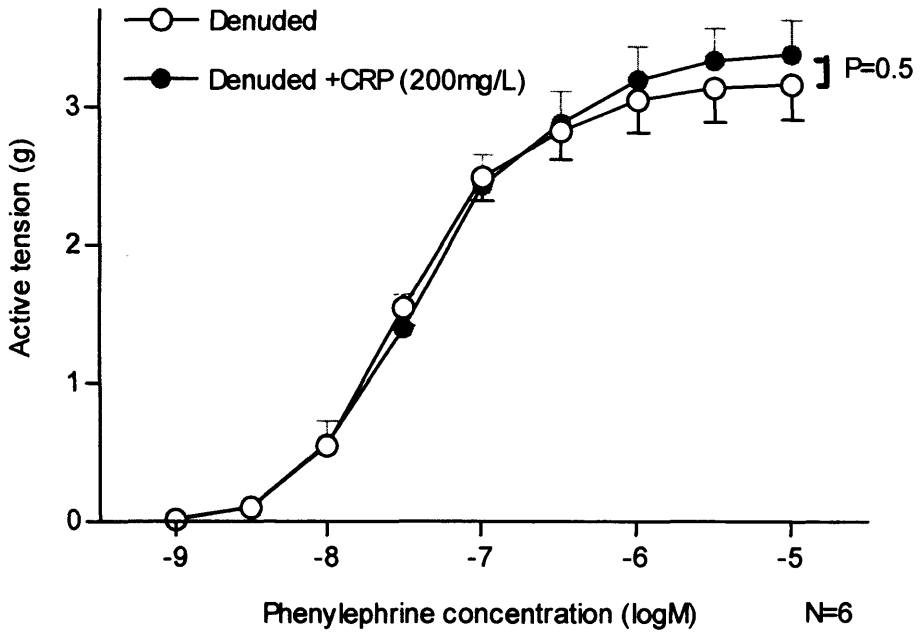



**Figure 6-7: Concentration response curve for rat aortic rings showing hyporeactivity to phenylephrine induced by CRP (•) relative to control (○) is abolished by endothelial denudation (N=6; P=0.5 for CRP vs Control). Contemporaneous positive and negative controls have been removed for clarity (P<0.001 for CRP vs control with intact endothelium).**

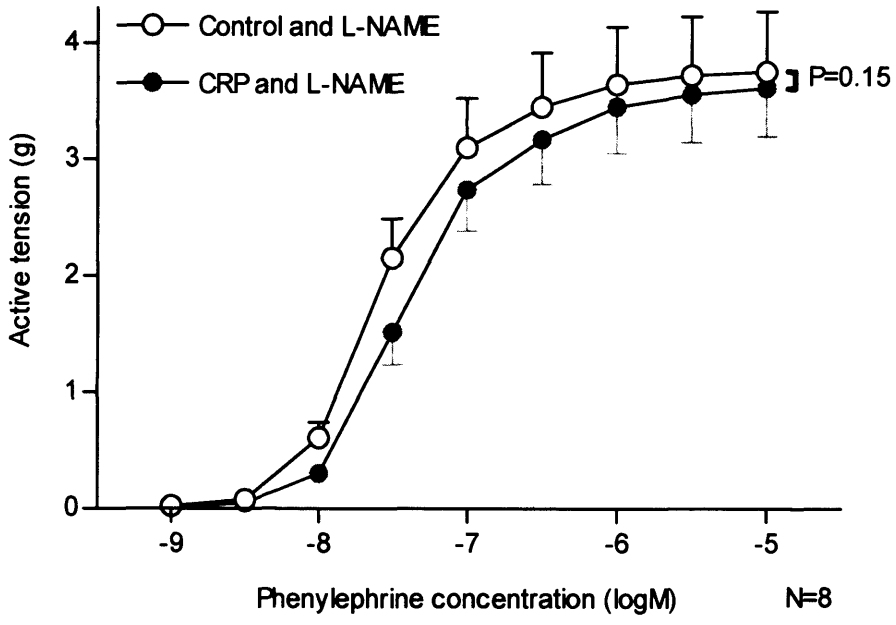



**Figure 6-8: Concentration response curve for rat aortic rings showing hyporeactivity to phenylephrine induced by CRP (•) relative to control (○) is abolished by incubation with L-NAME ($3x10^{-4}$M) (N=8; P=0.15 for CRP vs Control). Contemporaneous positive and negative controls have been removed for clarity (P<0.001 for CRP vs control without L-NAME).**

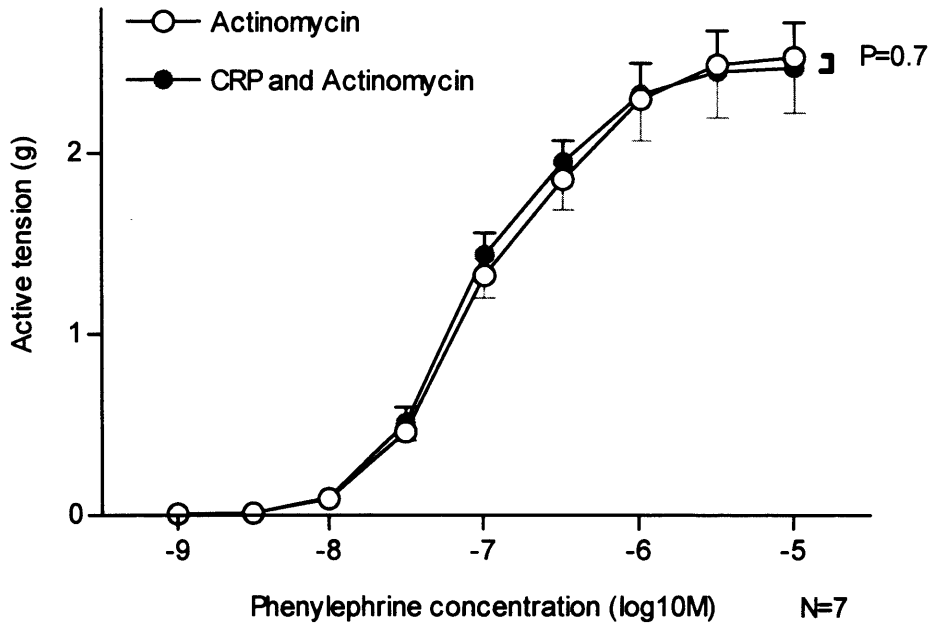



**Figure 6-9: Concentration response curve for rat aortic rings showing hyporeactivity to phenylephrine induced by CRP (•) relative to control (○) is abolished by incubation with actinomycin D ($10^{-5}$M) (N=7; P=0.7 for CRP vs Control). Contemporaneous positive and negative controls have been removed for clarity (P=0.002 for CRP vs control without actinomycin).**

### 6.3.2 CRP augments NO availability in the absence of iNOS induction

To exclude a contribution from iNOS-derived NO additional studies were performed with 1400W, a specific inhibitor of iNOS. In contrast to L-NAME, 1400W failed to reverse the PE hyporeactivity induced by CRP (Figure 6-10).

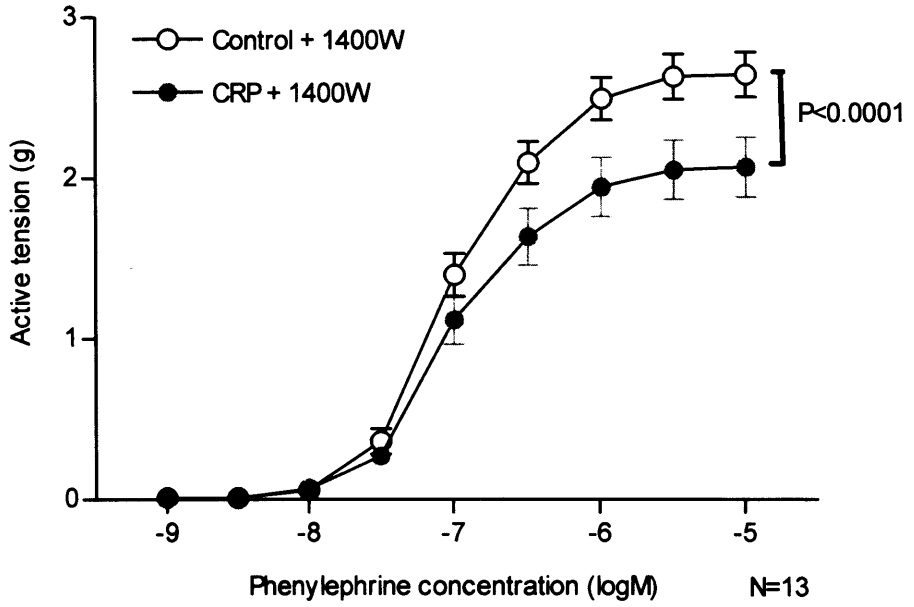



**Figure 6-10: Concentration response curve for rat aortic rings showing hyporeactivity to phenylephrine induced by CRP (•) relative to control (○) is preserved after incubation with 1400W ($10^{-5}$M) (N=13; P<0.0001 for CRP vs Control). Contemporaneous positive and negative controls have been removed for clarity (P<0.001 for CRP vs control without 1400W).**

In keeping with an iNOS-independent mechanism, in rings pre-contracted with phenylephrine, there was no difference in the relaxation to L-arginine (or its control D-arginine) in CRP-treated vessels compared to those left untreated (Figure 6-11).

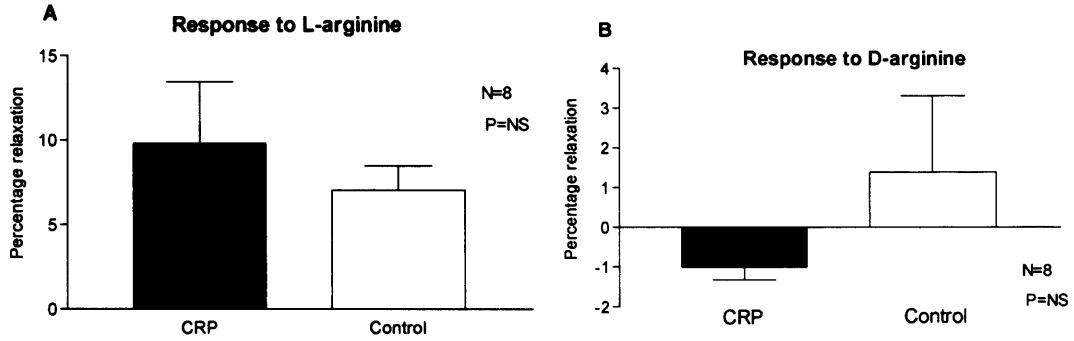



**Figure 6-11: Percentage relaxation to L-arginine (A) and D-arginine (B) in partially pre-constricted vessel rings is not effected by pre-treatment (N=8, P=NS).**

In control experiments 1400W entirely reversed the hyporeactivity to PE induced by incubation with lipopolysaccharide, which is known to be iNOS-dependent. In vessels that were not pre-incubated for 4 hours 1400W treatment did not lead an increase in the response to PE. This indicates that 1400W can inhibit the expected iNOS dependent response of LPS and that the incubation process leads to a small degree of iNOS induction independent to the treatment group.

### 6.3.3 CRP augments NO availability as a result of increased synthesis of tetrahydrobiopterin

In rat aortic rings incubated with CRP and pre-contracted with PE, there was a small but significant increase in the sensitivity to the endothelium-dependent vasodilator ACh (Figure 6-12: P<0.0001) with no change in the response to the endothelium-independent vasodilator SNP (Figure 6-13), consistent with increased NO availability.

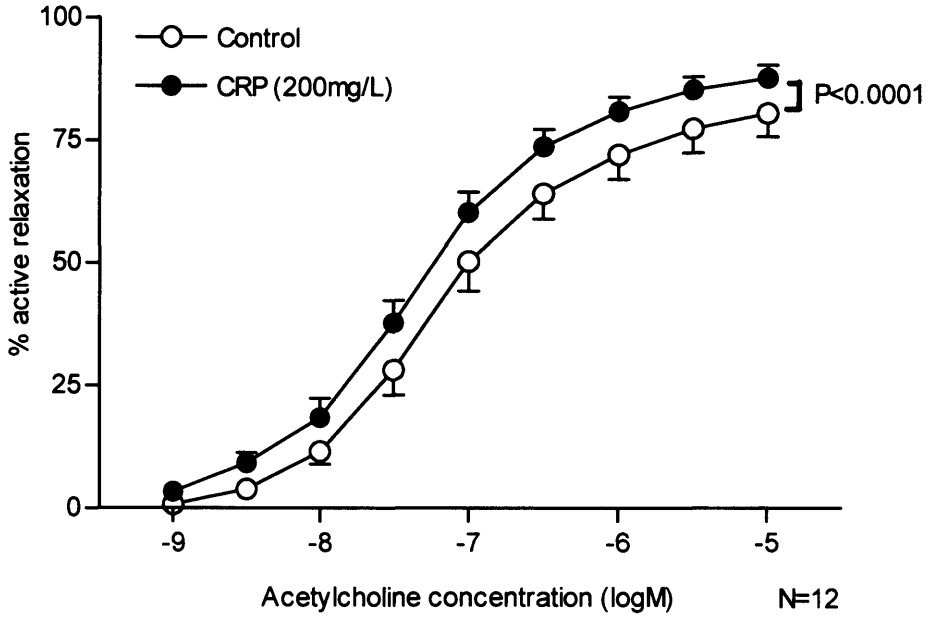



**Figure 6-12: Concentration response curve for rat aortic rings showing increased sensitivity to acetylcholine induced by CRP (•) relative to control (○) (N=12; P<0.0001 for CRP vs Control).**

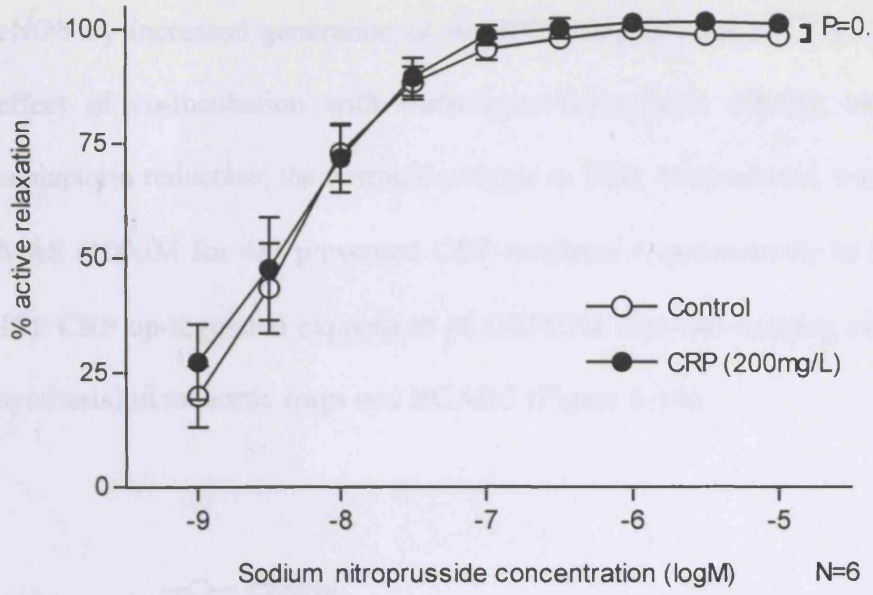



**Figure 6-13: Concentration response curve for rat aortic rings showing similar sensitivity to sodium nitroprusside with CRP (●) treatment and control (○) (N=6; P=0.1 for CRP vs Control).**

Despite the functional increase in NO availability and the requirement for new protein synthesis, CRP did not alter eNOS expression in rat aortic rings (not shown) or HCAECs in culture (Figure 6-14).

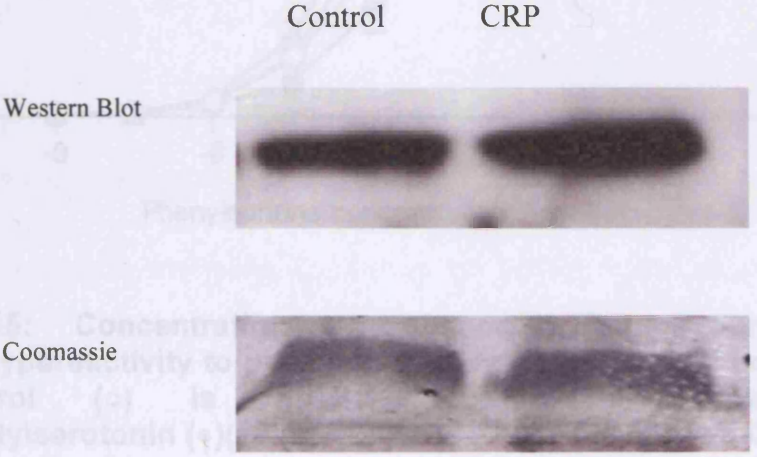



**Figure 6-14: Western blot of eNOS expression by human coronary artery endothelial cells incubated with CRP (50mg/L, chosen for reasons of dilution) or control for 24 hours showing no difference. Coomassie stain shows equal protein transfer.**

NO availability can be increased in the absence of transcriptional induction of eNOS by increased generation of the NOS cofactor $BH_4$([323;376]). Therefore the effect of co-incubation with methoxyacetylserotonin (MAS), an inhibitor of sepiapterin reductase, the terminal enzyme in $BH_4$ biosynthesis, was investigated. MAS (100μM for 4h) prevented CRP-mediated hyporeactivity to PE (Figure 6-15). CRP up-regulated expression of GTPCH1 (the rate-limiting enzyme in $BH_4$ synthesis) in rat aortic rings and HCAEC (Figure 6-16).

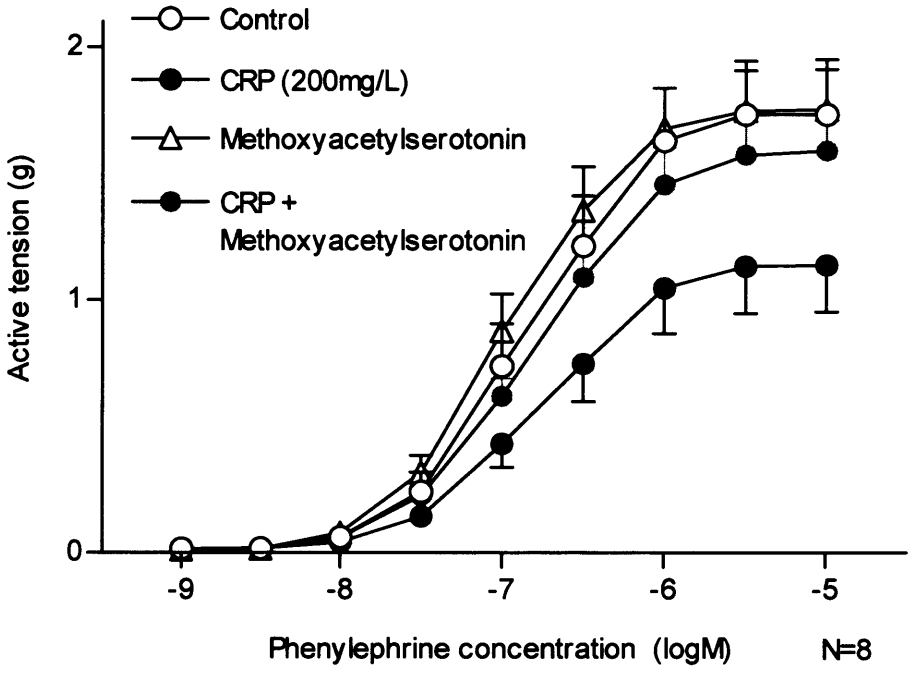



**Figure 6-15: Concentration response curve for rat aortic rings showing hyporeactivity to phenylephrine induced by CRP (•) relative to control (○) is abolished after incubation with methylacetylserotonin (•)($10^{-4}$M) (N=8; P<0.0001 for CRP vs Control).**

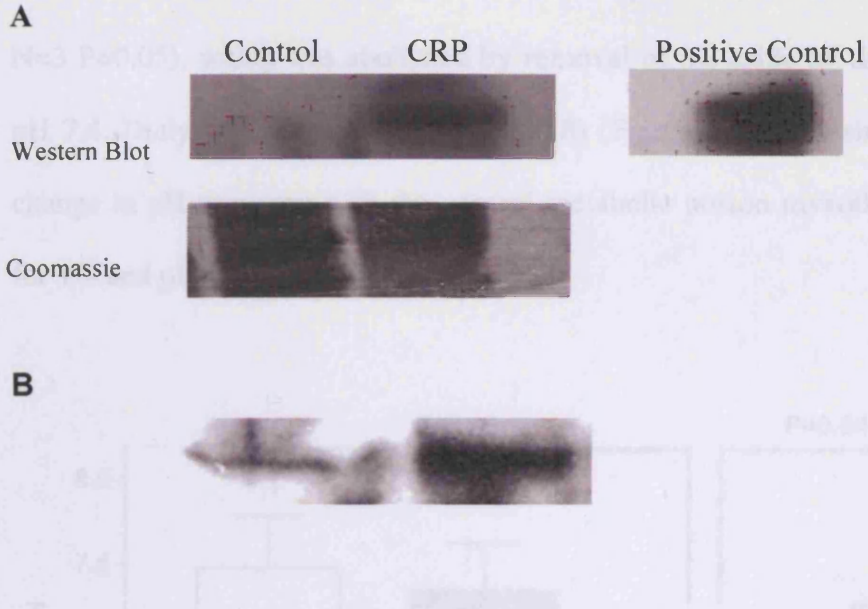



**Figure 6-16: A) Western blot of GTPCH1 expression by human coronary artery endothelial cells incubated with CRP (50mg/L) or control for 24 hours showing upregulation in the CRP treated cells. Positive control of porcine kidney. Coomassie stain shows equal protein transfer.**

**B) Western blot for GTPCH1 expression in rat tissue after incubation with 200mg/L CRP for 4 hours against control. Coomassie stain assessment of transfer equal (not shown).**

### 6.3.4 Effect of CRP in mouse endothelial cell model

In these experiments, involving incubation of GFRP overexpressing mouse endothelial cells with various CRP preparations, only treatment with the positive control of LPS, IL-1Ra and TNF led to any significant change in NO production. In the case of this positive control NO production was reduced in GFRP over-expressing cells, with no significant effect seen in other groups. In the experiments to detect the effect of incubation with different CRP preparations upon pH there was no difference between control and azide-free CRP (Control pH

7.4, CRP pH 7.1; N=3 P=0.5). However there was a significant reduction in pH in cells incubated with commercial CRP (Control pH 7.4, Calbiochem CRP pH 6.6; N=3 P=0.05), which was abolished by removal of the azide by dialysis (Control pH 7.4, Dialysed CRP pH 7.2; N=3 P=0.8) (Figure 6-17). A similar degree of change in pH was seen with the control metabolic poison myxothiazole (pH 6.3 for 1% and pH 6.9 for 0.1%).

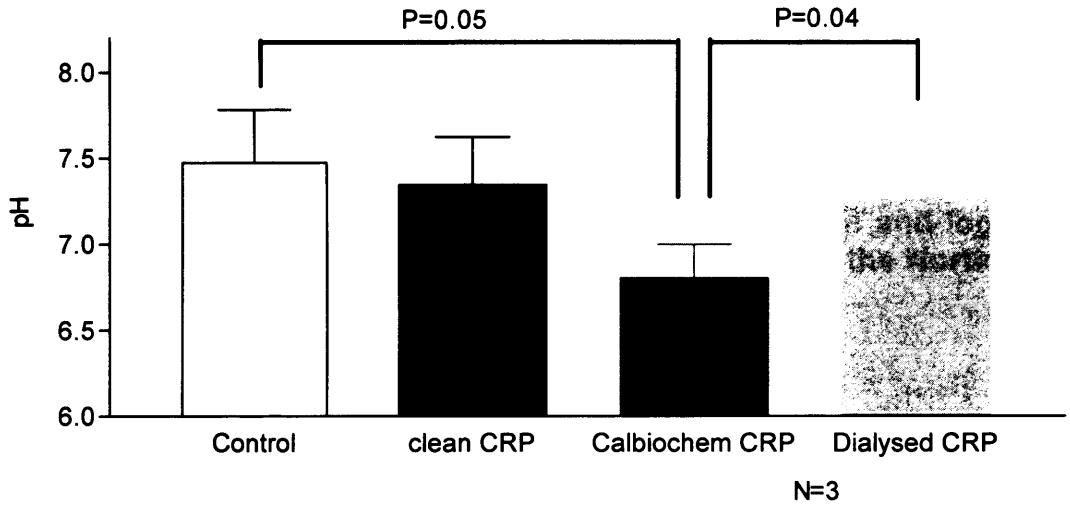



**Figure 6-17: Changes in pH of culture media following incubation with different CRP preparations for 24 hours. There is a significant reduction in the pH of Calbiochem CRP treated cells compared to control that is abolished by removal of sodium azide.**

### 6.3.5 Plasma concentrations of CRP correlate with those of neopterin in the absence of clinically apparent inflammation

In 579 healthy middle-aged men from the Northwick Park Heart Study evaluated in the absence of clinically apparent infection or inflammation, plasma CRP concentration was correlated with plasma neopterin, an index of pterin pathway activation ($r^2$=0.012, P=0.007) (Figure 6-18).

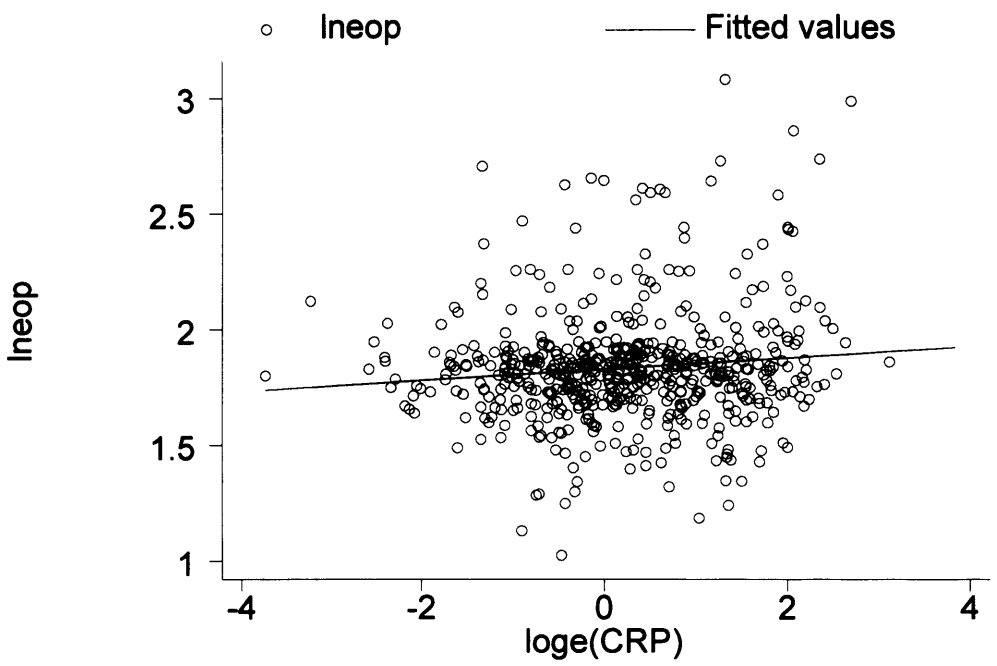



**Figure 6-18: Correlation between log plasma neopterin and logCRP concentrations in a large cohort of healthy males from the Northwick Park Heart Study. (N=579, $r^2$=0.012, P=0.007)**

## 6.4 Discussion

CRP is well characterised as an acute phase reactant that may have an important role in host defence against infection. Measurement of CRP has been widely used in clinical practice to assess the severity and monitor the progress of infective or inflammatory disorders but, more recently, CRP concentrations within the range found in health have been shown to be strongly predictive of future cardiovascular events. This has led to the proposal that CRP might itself play a role in atherogenesis, a proposal supported by data from a small number of studies that have indicated that CRP might contribute to the development of endothelial dysfunction([311;312]).

This study investigated the direct effects of CRP on vascular cells and tissues and, contrary to several previous reports([298;299]) , showed that CRP increases rather

than decreases NO-bioavailability in blood vessels. This is indicated, in the first instance, by the impairment in contractility to PE. This was in the form of "functional antagonism", that is a reduction in vasoconstriction being caused by an excess of vasodilators. That the excess vasodilator was nitric oxide is indicated by the ability of L-NAME, a non-specific NOS inhibitor, to restore PE-induced vasoconstriction to normal. The ability of NOS inhibition to return the vessel to normal contractility also indicated that there was no intrinsic abnormality in the vascular response to the vasoconstrictor, supporting the concept of functional antagonism.

NO can be generated from three NOS isoforms and in models of sepsis a similar hyporeactivity to PE was observed due to iNOS induction by LPS([488]). In contrast, in these experiments the source of the additional NO appeared to be eNOS. This was supported by the prevention of hyporeactivity by endothelial denudation, the failure of the iNOS-specific inhibitor 1400W to abolish the effect and the failure of L-arginine to cause significant relaxation (which does occur with iNOS induction). Importantly eNOS was also implicated by the enhanced relaxation seen to the endothelial dependent vasodilator (ACh), with no parallel increase in response to the NO donor SNP. These effects of CRP were seen in human and rat blood vessels, over a range of physiological and pathophysiological concentrations of CRP.

Though requiring new protein synthesis, as indicated by the prevention of hyporeactivity when new protein synthesis was prevented by incubation with actinomycin, these actions of CRP were independent of increased eNOS

expression by western blotting. Instead, CRP led to an induction in the expression of GTPCH-1, the rate-limiting enzyme in the synthesis of $BH_4$. The functional importance of this increase in $BH_4$ was demonstrated by the amelioration of the PE hyporeactivity after incubation with the pterin pathway inhibitor MAS. Similarly in the epidemiological human data there was an association between CRP and neopterin concentrations, though this needs to be treated with caution, as neopterin is itself acute phase reactant and therefore the association between CRP and neopterin could be confounded.

Our group, and others, have shown previously that increases in the concentration of $BH_4$ mediated by inflammatory cytokines([323]), or estrogens([376]), can lead to increases in NO synthesis from eNOS, independent of changes in enzyme expression. Failure to show any effect on NO production in the mouse cell culture model could reflect a number of points. Firstly the mouse cells may not respond in the same way to human CRP, due to a cross-species effect, and secondly the degree of NO production upregulation may not be adequate to be detected by the relatively insensitive Greiss assay.

In contrast to the current findings, previous cell culture studies have identified increased endothelial cell adhesion molecule expression and down-regulation of eNOS expression and activity following incubation with CRP, in line with a pro-atherogenic rather than a protective role([298;299]). How can this difference be reconciled? While it is possible that differences in the concentrations of CRP studied, or the time course of exposure may play a part in the *in vitro* experiments, one important difference is the source of CRP.

In many previous studies, CRP has been obtained from commercial sources([290;298]). Such preparations usually contain significant concentrations of sodium azide (0.05%w/v; final concentration 370μM), which is known to have important vasoactive effects by the generation of nitric oxide from its catalase-dependent breakdown([276-278]). This exogenous production of NO could then lead to down-regulation of NO production by eNOS([489]). A recent paper with CRP preparations in which the azide had been removed by dialysis indicated that the acute vasodilator effect of commercially sourced CRP is by way of this direct NO-donor effect([300]). Sodium azide also alters cell metabolism by inhibiting the mitochondrial respiratory chain and therefore will favour anaerobic respiration and the development of an acidosis([278]). This can be seen in the experiments with mouse cell lines where incubation with commercial CRP lead to a significant fall in pH of the culture medium. To overcome this problem, CRP was prepared to a high degree of purity, free from sodium azide.

A number of previous studies have failed also to confirm the specificity of observed effects by the use of appropriate controls. In the current study, the vascular effects of CRP were abolished by pre-absorption of the CRP with phosphocholine-coated sepharose beads and then incubation of the resultant solution in a blinded fashion. Further, a specific inhibitor of CRP-phosphocholine-ligand binding (RMM) abolished the CRP-induced PE hyporeactivity. Finally, it was confirmed that the effects observed could not be explained by contamination of solutions by bacterial endotoxin. As such this work has attempted to show an effect that is robustly attributable to CRP and not

contaminants. Caution must be applied to the effects of one species of CRP on another species vessel, in particular because CRP has different background levels and immunological effects across species. This was addressed by repeating some of the experiments in isolated human vessels and human cells lines.

Although these experiments have explored the effects of CRP on NO bioavailability in some detail alternative modes of action cannot be excluded. CRP could act multifactorially to alter substrate availability, though in this case an effect on L-arginine supplementation would be expected. Alternatively it might alter ADMA concentrations or activity, influence to generation of reactive oxygen species, or alter post-translation enzymic structure (for instance by phosphorylation). These areas have not been specifically addressed in this work.

In summary, short-term exposure of vascular cells or tissues to highly purified human CRP results in induction of NO synthesis from eNOS, probably via increased provision of the NOS co-factor $BH_4$. These actions point to a potentially protective role for CRP in the resolution of endothelial dysfunction that may follow acute sepsis or inflammation, or during the low-grade inflammation that exists during the long pre-clinical phase of atherosclerosis. Interestingly this ties in with the data in the first chapter that shows the time course of the development of a significant CRP concentration coincides with the resolution, rather than development, of endothelial dysfunction. It is important to note that these observations do not undermine the utility of CRP measurement in the prediction of future disease, which is based on the demonstration of a strong association in many large epidemiological studies. The development of selective

inhibitors of CRP binding will aid future studies addressing the physiological and pathological properties of CRP.

# 7 Conclusion

This thesis has explored the effects of inflammation of vascular function *in vivo* and *in vitro*. A number of themes have been developed and this section will bring these together.

## 7.1 Methodological issues

There is an increasingly large literature on the direct vascular effects of CRP. Almost all of this uses the commercially sourced CRP, which is intended as a reference standard for assaying the accuracy of diagnostic tests. An important aspect of this thesis has been the appreciation of the biological importance of the agents added to this CRP. In particular, sodium azide is included to prevent the growth of bacterial contaminants, as it is a mitochondrial chain inhibitor, to prevent contamination of the agents. However the direct effects of the sodium azide on the biological system itself cannot be ignored. In the experiments described in section 6 not only was a highly purified CRP used, free from bacteriostatic agents, but also experiments were also performed to address the issue of specificity. As a result one can be confident that the effects demonstrated are as a result of the actions of the CRP and not any other agent in which it is dissolved.

Another important methodological issue addressed in this research is the difference between cross-sectional and longitudinal studies. Previous publications looking at an association between the serum concentration of CRP and the

presence of endothelial dysfunction failed to take into account the inflammatory milieu in which the CRP exists. Importantly in a cross-sectional study it is not possible to determine which of the various raised inflammatory mediators is important to the observed biological effects. This work used a time-course model to tease out the impact of some of the different mediators, particularly that the rise in CRP following an inflammatory stimulus is temporally dissociated from the induced endothelial dysfunction in this model. Although this alone does not allow for the attribution of causality it is more suggestive than is possible from a cross-sectional analysis alone.

## 7.2 Inflammation as a cause of endothelial dysfunction

The introduction developed the idea that endothelial dysfunction is important as a mediator of traditional risk factors in the development of atherosclerotic plaques and also their destabilisation to produce clinical events. Previous work by our group, and others, has suggested that inflammation may in a similar way be important in atherosclerosis, again by an action mediated through endothelial dysfunction. This work has taken these concepts further, by exploring the role an acute inflammatory stimulus has over a longer time period and the changes it produces in nitric oxide bioavailability.

Following vaccination there is a transient reduction in the bioavailability of NO, seen both in terms of a reduced effect of NOS inhibition and impairment in the flow-mediated response (thought largely to be NO mediated). These effects can be related to both the conduit (FMD) and resistance (L-NMMA effect) vasculature of the forearm and certainly in the case of FMD lasts less than 36

hours. All these studies were performed in healthy individuals and as such it is not possible to say whether these effects would be more marked or reduced in the presence of other risk factors.

How these changes appear to be mediated is discussed below. Although vaccination leads to the development of a reproducible cytokine response (IL-1Ra – IL6 – CRP) it is not clear as yet exactly which component is involved in the changes described. In contrast to some previous literature CRP does not appear to be the casual agent as its biological dynamics mean that it does not increase in level until after the development and recovery of the endothelial abnormality. To explore the impact of each individual mediator would require the use of specific inhibitors and the determination of whether each one alters the vascular responses. One of the ways in which inflammation, endothelial dysfunction and atherosclerosis has been linked is the association between CRP concentrations and future atherothrombotic events. This work however does not support the proposed causal role for CRP – does this nullify these previous associations? Probably not, as CRP can be viewed as a summative inflammatory mediator that reflects the presence of other, potentially causal, mediators. The biology of CRP makes it a very effective barometer of other inflammatory markers, which are often too transient to monitor. As such this work does not contradict the utility of the observation that CRP predicts future vascular events, just that it is unlikely to be causal.

## 7.3 Importance of reactive oxygen species to inflammation-induced endothelial dysfunction

Two of the data sections in this work look at the impact of inflammation on the degree of oxidant stress within the circulation. Reactive oxygen species have been implicated, by a number of authors, in the development and progression of atherosclerosis. This work has shown that a mild inflammatory insult can lead to the development of increased oxidant stress within the circulation. Further, this work indicates that reversal of this pro-oxidant state has different effects depending upon the point at which it is performed. After the development of endothelial dysfunction ascorbic acid is only partially effective. However, pre-treatment entirely avoids the development of vascular abnormalities. This raises the possibility that either ascorbic acid has different effects at different times, perhaps acting as a direct free radical scavenger acutely while a pre-exposure allows an action through other pathways (for instance $BH_4$).

It is not clear from this work exactly how oxidant stress is implicated in the development of endothelial dysfunction. Free radicals can directly breakdown NO and thereby reduce its bioavailability. However if this were the entire effect it would be expected that adequate quantities of ascorbic acid given at any point would entirely restore endothelial function. As this is not the case it increases the likelihood that the effect is at least in part mediated by an indirect mechanism. This would explain the partial effect of delayed asorbic acid and brings to the fore the role of NOS co-factors, in particular $BH_4$.

Interestingly the failure of delayed ascorbic acid to fully reverse the effects of inflammation on endothelial dysfunction may have implications for our understanding of clinical trials of vitamin supplementation in secondary prevention. If a similar effect is seen it may be that the supplements are given "too late" in the natural history of atherosclerosis to be effective. As such an early intervention study, most probably by dietary alteration, may produce a more favourable outcome.

## 7.4 Importance of tetrahydrobiopterin to the bioavailability of nitric oxide

In both the vaccine and *in vitro* CRP studies tetrahydrobiopterin assumes a central role. Vaccination leads to the development of relative $BH_4$ deficiency, indicated by the development of a dependence on $BH_4$ in the basal flow within the forearm vasculature. This effect is abolished by the pre-infusion of ascorbic acid, suggesting that inflammation may act to reduce $BH_4$ bioavailability by its oxidation to $BH_2$. The consequence of this would be a reduction in NO bioavailability and a further increase in oxidant stress. Changes in $BH_4$ are therefore the potential common pathway for the effects of inflammation on vascular function.

How does CRP fit into this model? Two observations from this work are important – firstly CRP appears to increase the production of $BH_4$ by means of an increase in the levels of the rate-limiting production enzyme (GTPCH-1) and secondly the rise in CRP concentration appears to coincide with the recovery of NO-dependent vascular responses. Perhaps CRP is not involved in the

development of endothelial dysfunction, as suggested by other authors, but rather implicated in the restoration of vascular intergrity following an inflammatory insult. This may be important in host defences as it allows the increase in flow to the damaged area that is required for effective healing.

## 7.5 Future work

This thesis has thrown up almost as many questions as it has attempted to answer. Some of the important ones are explored below, grouped according to methodology.

### 7.5.1 Vaccination studies

All of the work using this model has so far concentrated on healthy volunteers. However, the population most at risk of vascular events possess a number of conditions known to predispose to cardiovascular disease. It would be informative to study the impact of these risk factors (for instance smoking) on the magnitude and time course of the endothelial dysfunction to see if co-existant pathologies augment or diminish the observed effects.

This work has implicated changes in IL-6 and IL1-Ra in the development of the effects seen and this could be further detailed by selective antibody inhibition of these mediators. The role of tetrahydrobiopterin needs further exploration, particularly the relative importance of the different redox states of the co-factor. With the development of a robust assay the changes in $BH_4/BH_2$ ratios over the time course of the vascular effects following vaccination could be quantified.

Although the L-NMMA studies indicate that there is a reduction in basal nitric oxide bioavailability following vaccination, they do not confirm that the reduction in response to bradykinin is only mediated through a NO-dependent mechanism. Bradykinin may cause vasodilitation by changes in prostaglandins or EDHFs. The relative importance of each potential pathway could be explored by generating bradykinin dose response curves in the presence of basal NOS inhibition (with L-NMMA) and separately prostaglandin inhibition (with indomethacin).

### 7.5.2 Clinical studies

The work described in this thesis used a model of inflammation to explore the possible mechanisms by which endothelial dysfunction is induced. The rationale for this approach is that there is an increase in cardiovascular events following intercurrent illnesses and that endothelial dysfunction may act as the link between these observations. All models have their limitations and it would be extremely interesting to explore the effects of a clinically relevant inflammatory stimulus (such as surgery). This could be achieved by measuring FMD responses in the forearm of patients undergoing abdominal surgery, before, during and for a period after the operation and comparing this to changes in inflammatory mediators.

Having established this clinical model the mechanism of any changes seen could be explored. In particular it would be useful to see if pre-treatment with ascorbic acid in any way protected the individual. The route of administration of ascorbic acid could be varied to determine whether it needs to be given systemically. In an

adequately powered study the patients could be followed up to determine whether the frequency of clinical vascular complications following the surgery correlate with the extent of changes in endothelial function. In an intervention study it could also be seen whether pre-treatment of surgical patients with anti-oxidants, or perhaps $BH_4$, altered the frequency of cardiovascular events.

The studies in this work explore changes in vascular reactivity in the peripheral circulation and assume, as other studies indicate, that these act as an effective model for coronary and cerebral vascular responses. It would be of interest, though practically difficult, to determine the actual changes in coronary vasculature following an inflammatory insult. Although this could be performed in a cross-sectional way with correlations made to measured inflammatory indices, it would be preferable to measure the vascular reactivity at two time points, one acute and one following recovery from the index event. There are obviously practical and ethical issues surrounding repeated invasive studies and this type of work would best be added onto another study that already requires serial measurements.

### 7.5.3 *In vitro* studies

In the *in vitro* studies in this thesis it was assumed that any effect of CRP on vascular function would take a time to occur. This was supported by the observation that new protein synthesis is required for the changes in vascular responses to develop. However, to date it is not clear exactly the duration of incubation needed to produce an effect and whether there may be different effects dependent on the time for which tissues are exposed to CRP. This could be

explored in two ways, either organ bath experiments could be performed after a series of different incubation periods (for example 1 hour, 2 hours, 4 hours, 8 hours and 16 hours) or the CRP could be added to tonically contracted vessels already mounted in the organ bath and monitor the time to vessel relaxation. At the end of this experiment the addition of NOS inhibitors could show that the effect seen is NO-dependent.

From the work in this thesis it seems possible that CRP exerts its cardiovascular effects by changes in $BH_4$ or the $BH_4/BH_2$ ratio. It would be informative to directly measure the impact of CRP on the concentration of these co-factors both in *in vitro* and *in vivo* experiments. As part of this it would be interesting to determine the effect of instilling highly purified CRP into human vessels *in vivo*. This would best be achieved with a hand vein model where the vein is isolated from the circulation before the CRP is instilled and its vascular responses determined. Similar experiments using cytokines have been previously performed by our group([323]).

## 7.6 Final comment

This thesis describes a body of work aiming to explore the mechanisms by which inflammation can lead to endothelial dysfunction. The studies have shown the importance of oxidant stress in this process and the potentially central role of tetrahydrobiopterin. The vascular effects of the acute phase protein CRP have been explored in great detail, both *in vivo* and *in vitro*. During this process I have learnt a large amount about the design, execution and interpretation of well-controlled scientific experiments. Further studies, indicated in the section above, will hopefully allow the application of these observations to clinically relevant

inflammatory insults and ultimately lead to the development of novel therapeutic strategies to reduce morbidity and mortality.

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